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EGFR Plus RGQ PCR Kit

Instructions for Use



Version 1

For use with plasma or FFPE tissue
For the detection of 42 mutations in the *EGFR* gene
For use with Rotor-Gene® Q instrument
For Research Use Only. Not for use in diagnostic procedures.

REF

874601



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Store at -30 to -15°C

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Intended Use

The EGFR Plus RGQ PCR Kit is a research use only real-time PCR test intended for the qualitative detection and identification of mutations in exons 18, 19, 20, and 21 of the epidermal growth factor receptor (*EGFR*) gene (1) in DNA samples extracted from plasma or formalin-fixed paraffin-embedded (FFPE) tumor tissue.

The EGFR Plus RGQ PCR Kit is further intended for the semi-quantitative measurement of somatic mutations in exons 18, 20, and 21 of the *EGFR* gene in DNA samples extracted from plasma.

The EGFR Plus RGQ PCR Kit is to be used by trained personnel in a professional laboratory environment for research use only. It is not intended for diagnostic procedures.

Intended User

This kit is intended for professional use.

The product is to be used only by personnel specifically instructed and trained in molecular biology techniques and familiar with this technology.

Description and Principle

Summary and explanation

The EGFR Plus RGQ PCR Kit is a real-time PCR (polymerase chain reaction) test for the detection of 42 mutations in the *EGFR* gene using ARMS (Amplification Refractory Mutation System) (2, 3) and PCR clamp technologies for the qualitative detection and identification of mutations in the EGFR gene; exons 18, 19, 20, and 21 (Table 1). The kit allows the semi-quantification of G719X (with X = A, S or C; exon 18), T790M (exon 20), C797Sa and C797Sb (exon 20), S768I (exon 20), L858R (exon 21), and L861Q (exon 21) in DNA samples extracted from plasma. In summary:

- G719X in exon 18 (detects and semi-quantifies G719S, G719A, or G719C, but does not distinguish between them)
- 28 deletions in exon 19 (detects the presence of any of the 28 deletions but does not distinguish between them)
- S768I, T790M, C797Sa and C797Sb in exon 20 (detects and semi-quantifies all four mutations, but does not distinguish between C797Sa and C797Sb)
- Five insertions in exon 20 (detects the presence of any of the five insertions, but does not distinguish between them)
- L858R and L861Q in exon 21 (detects and semi-quantifies the two mutations)

The methods used are highly selective and, depending on the total amount of DNA present, enable detection of a low percentage of mutant DNA in a background of wild-type genomic DNA. These selectivity and detection limits can be compared to technologies such as dyeterminator sequencing.

Table 1. List of mutations and COSMIC identities

Exon	Mutation	COSMIC ID*	Base change
18	G719A	6239	c.2156G>C
	G719S	6252	c.2155G>A
	G719C	6253	c.2155G>T
19	Deletions	26038	c.2233_2247del15
		13550	c.2235_2248>AATTC
		6223	c.2235_2249del15
		6225	c.2236_2250del15
		18427	c.2237_2257>TCT
		6220	c.2238_2255del18
		12367	c.2237_2254del18
		12384	c.2237_2255>T
		12678	c.2237_2251del15
		13551	c.2235_2252>AAT
		13552	c.2235_2251>AATTC
		12386	c.2237_2252>T
		12416	c.2237_2253>TTGCT
		12728	c.2236_2253del18
		12422	c.2238_2248>GC
		12382	c.2239_2248TTAAGAGAAG>C
		6218	c.2239_2247delTTAAGAGAA
		12387	c.2239_2258>CA
		12370	c.2240_2257del18
		12403	c.2239_2256>CAA
		6255	c.2239_2256del18
		12383	c.2239_2251>C
		12419	c.2238_2252>GCA
6210	c.2240_2251del12		
23571	c.2238_2252del15		
12369†	c.2240_2254del15		

		13556	c.2253_2276del24
		12385	c.2235_2255>AAT
20	S768I	6241	c.2303G>T
	Insertions	12376	c.2307_2308insGCCAGCGTG
		12378	c.2310_2311insGGT
		12377	c.2319_2320insCAC
		13428	c.2311_2312insGCGTGGACA
		13558	c.2309_2310AC>CCAGCGTGGAT
	T790M	6240	c.2369C>T
	C797Sa	6493937	c.2389T>A
	C797Sb	5945664	c.2390G>C
21	L858R	6224	c.2573T>G
	L861Q	6213	c.2582T>A

* COSMIC: Catalogue Of Somatic Mutations In Cancer: <http://cancer.sanger.ac.uk/cosmic>

† According to the new COSMIC database, due to the similarities in sequence after the deletion has occurred, deletion 6254 is combined with deletion 12369.

Principle of the procedure

The EGFR Plus RGQ PCR Kit uses real-time PCR for the detection of 42 mutations in the *EGFR* gene (exons 18, 19, 20, and 21) and the semi-quantification of G719X (with X = A, S or C; exon 18), T790M (exon 20), C797Sa and C797Sb (exon 20), S768I (exon 20), L858R (exon 21), and L861Q (exon 21). The EGFR Plus RGQ PCR Kit will test genomic DNA (gDNA) extracted from FFPE tumor tissue and circulating cell-free DNA (ccfDNA) extracted from plasma samples using the Rotor-Gene® Q 5plex HRM instrument. Data analysis and result interpretation are performed manually using Rotor-Gene Q software version 2.3 or later and the instructions provided in this Instructions for Use (Figure 1).

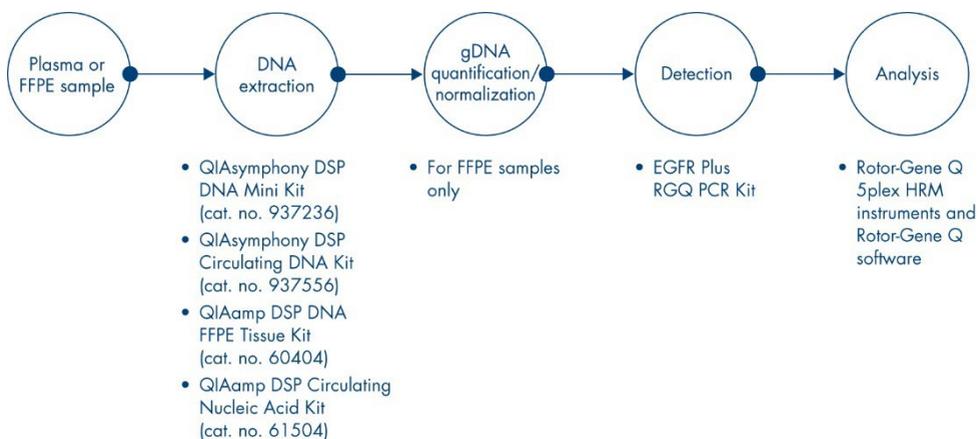


Figure 1. EGFR Plus RGQ PCR Kit workflow. For non-DSP extraction kits, see “Materials Required but Not Provided”, page 21.

Technology

The use of qPCR permits the accurate detection of PCR products during the exponential phase of the PCR amplification process. qPCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during PCR cycling.

The EGFR Plus RGQ PCR assays exploit the qPCR oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. Another dye-linked oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream 3' dye-free quencher, hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes exploits the 5'→3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer.

During PCR, if the target of interest is present, both forward and reverse primers specifically anneal and flank the probe. The 5'→3' exonuclease activity of the DNA polymerase cleaves

the probe between the reporter and the quencher only if the 3 oligonucleotides hybridize to the target. The probe fragments are then displaced from the target and polymerization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR (Figure 2). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

The increase in fluorescence signal is detected only if the target sequence is complementary to the primers and probe and hence amplified during PCR.

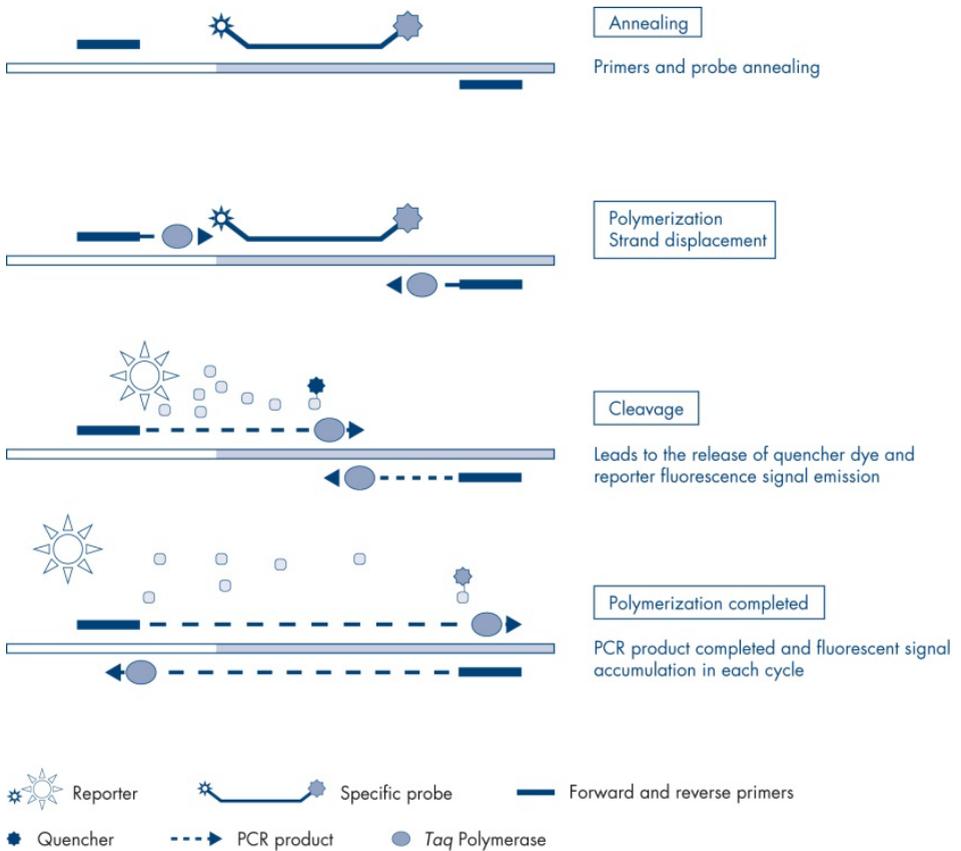
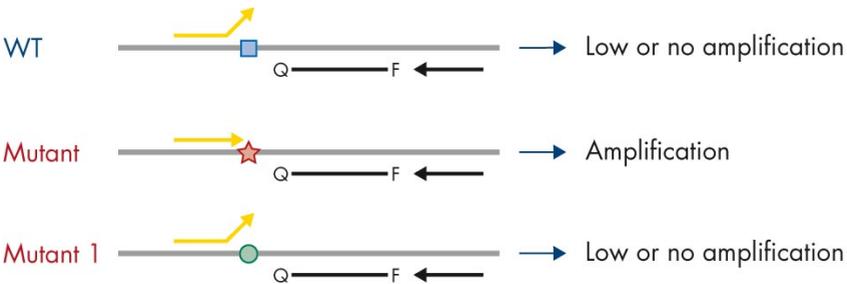


Figure 2. Reaction principle.

In the EGFR Plus RGQ PCR Kit, mutation-specific reactions use ARMS (amplification-refractory mutation system) and clamp designs to detect, identify, and semi-quantify (when applicable) mutations in DNA extracted from FFPE and plasma.

ARMS

Amplification-refractory mutation system (ARMS) exploits the ability of *Taq* DNA polymerase to distinguish between a matched and a mismatched base at the 3' end of a PCR primer. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low-level background amplification may occur. Therefore, a mutated sequence is selectively amplified, even in samples where the majority of the DNA does not carry the mutation (Figure 3).



■ WT sequence ★ Targeted mutant sequence ● Other mutant sequence

Figure 3. Identification of specific mutation by ARMS PCR. WT: Wild type. Q-F: Double-dye probe. D: Forward and reverse primers.

PCR clamp

This method is used to detect several variants localized in the same hotspot (e.g., *EGFR* deletions in exon 19). The clamp assay combines standard primers and probe with an additional oligonucleotide that is 3'-blocked by the addition of a phosphate group, in order to prevent PCR elongation. The clamp oligonucleotide, as well as the primers and probe, are specific to the wild-type sequence (PCR clamping). When the PCR template contains the wild-type sequence, the clamp hybridizes before the primer, due to a higher T_m leading to no or low amplification. In contrast, when a mutated sequence is present, the clamp cannot bind, which allows primer annealing and amplification (Figure 4).

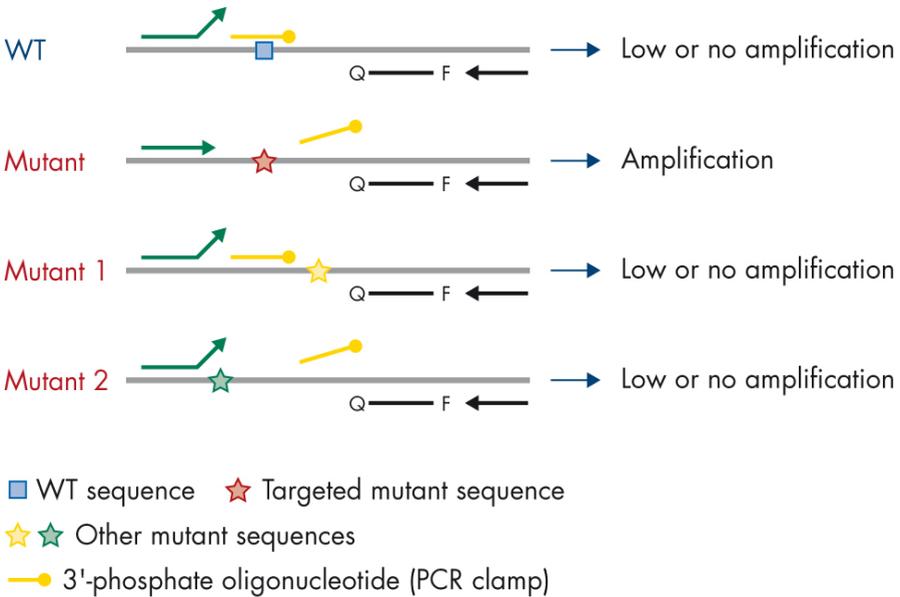


Figure 4. Mutation detection by clamp technology. WT: Wild type. Q-F: Double-dye probe. D: Forward and reverse primers.

Kit format and assays

Mutation assays

Four primer and probe mixes are supplied in the EGFR Plus RGQ PCR Kit:

- T790M and L861Q
- Insertions (exon 20) and G719X
- L858R and C797S
- Deletions (exon 19) and S768I

All primer and probe mixes, when combined with the PCR Master Mix, allow the detection of targets that are labeled with carboxyfluorescein (FAM™), CAL Fluor® Red 610 and an internal control labeled with hexachlorofluorescein (HEX™).

Internal control assay

The internal amplification control reaction, labeled with HEX, is used to assess the total amplifiable *EGFR* DNA template in a mutated and a non-mutated (wild type) sample (Figure 5), and to identify reaction failures due to suboptimal DNA input, or the presence of inhibitory substances in the sample matrix. This internal amplification reaction amplifies a region of exon 2 of the *EGFR* gene. The primers and probe have been designed to avoid any known *EGFR* polymorphisms.



■ WT sequence ★ Targeted mutant sequence

Figure 5. EGFR exon 2 internal control (IC) detection. WT: Wild-type. Q-F: Double-dye probe. D: Forward and reverse primers.

Water for sample dilution (Dil.)

The EGFR Plus RGQ PCR Kit contains Nuclease-Free Water to be used for gDNA sample dilution.

Controls

Each PCR run must contain a positive control (PC) and a negative control (NTC) for each of the four assays.

Positive control (PC)

Each run must contain a positive control in tubes 1–4. The EGFR Plus RGQ PCR Kit contains one *EGFR* Positive Control (PC) to be used as the template in the positive control reaction. The positive control results will be assessed to ensure that the kit performs within the predefined acceptance criteria.

No template control (NTC)

Each run must contain a negative control (no template control; NTC) in tubes 5–8. The EGFR Plus RGQ PCR Kit contains water for the NTC to be used as the “template” for the no template control. The no template control is used to assess any potential reagent and environment contamination.

Materials Provided

The contents of the EGFR Plus RGQ PCR Kit are sufficient for 24 samples (the kit contains sufficient reagents for up to four qPCR runs of six samples per run, including run controls).

Kit contents

EGFR Plus RGQ PCR Kit	(24)
Catalog no.	874601
Number of reactions	24

Color	Identity	Tube ID	Volumes
Green	T790M & L861Q Mix	<i>EGFR</i> T790M & L861Q Mix	280 µL
Yellow	Insertions & G719X Mix	<i>EGFR</i> Insertions & G719X Mix	280 µL
Purple	L858R & C797S Mix	<i>EGFR</i> L858R & C797S Mix	280 µL
Orange	Deletions & S768I Mix	<i>EGFR</i> Deletions & S768I Mix	280 µL
Red	EGFR Positive Control	<i>EGFR</i> Positive Control	190 µL
Blue	PCR Master Mix	<i>EGFR</i> PCR Master Mix	2 × 940 µL
Colorless	Water for NTC	NTC	1.9 mL
Colorless	Water for sample dilution	Dil.	1.9 mL

Platform and software

The EGFR Plus RGQ PCR Kit is specifically designed to be used with Rotor-Gene Q instruments* with fluorescent channels for cycling green, yellow, and red with Rotor-Gene Q software version 2.3 or later in Open mode.

* Ensure that instruments and equipment have been checked and calibrated according to the manufacturer's recommendations.

Materials Required but Not Provided

Additional reagents

- Deparaffinization Solution (cat. no. 19093 or 939018) for manual and automated gDNA preparation from FFPE samples
 - QIASymphony® DSP DNA Mini Kit (cat. no. 937236)* for automated gDNA preparation from FFPE samples
 - QIASymphony DSP Circulating DNA Kit (cat. no. 937556) for automated ccfDNA preparation (from plasma)
 - QIAamp® DSP DNA FFPE Tissue Kit (cat. no. 60404)† for manual gDNA preparation from FFPE samples
 - QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504)‡ for manual ccfDNA preparation from plasma samples
- Note:** Materials required but not provided for the DNA extraction kits listed above are detailed in the respective kit handbooks.
- RNase A (cat. no. 19101) for manual or automated gDNA sample preparation from FFPE samples
 - Buffer ATL (cat. no. 939016) for the deparaffinization protocol used with the QIASymphony DSP DNA Mini Kit (cat. no. 937236) or QIASymphony DNA Mini Kit (cat. no. 931236)

* The QIASymphony DNA Mini Kit (cat. no. 931236) can be used if the QIASymphony DSP DNA Mini Kit (cat. no. 937236) is not available.

† The QIAamp DNA FFPE Tissue Kit (cat. no. 56404) can be used if the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) is not available.

‡ The QIAamp Circulating Nucleic Acid Kit (cat. no. 55114) can be used if the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) is not available.

Consumables and general laboratory equipment

- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR Reaction Mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA
- Nuclease-free, aerosol-resistant, sterile PCR pipette tips with hydrophobic filters (pipette tips with aerosol barriers are recommended to help prevent cross-contamination)
- Vortex mixer*
- Benchtop centrifuge* with rotor for 0.5 mL, 1.5 mL, and 2.0 mL reaction tubes (capable of attaining 13,000–14,000 rpm)
- DNase, RNase, DNA-free, sterile 1.5 or 2.0 mL microcentrifuge tubes for preparing DNA and PCR reaction mixes
- Strip Tubes and Caps, 0.1 mL, for the Rotor-Gene Q 5plex HRM instrument (cat. no. 981103 or 981106)
- DNA quantification instrument
- Sample tubes (e.g., 2 mL Sarstedt® tube [cat. no. 72.693]) for automated gDNA preparation (from FFPE blocks). Compatible primary and secondary tube formats are listed at www.qiagen.com/goto/dspdnakits
- Single-use, sterile scalpel for manual and automated gDNA preparation (from FFPE section on slide sample)
- Sterile phosphate-buffered saline (PBS, may be required for topping up plasma sample volumes)

* Prior to use, ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Equipment for automated sample preparations

- QIAAsymphony SP instrument* (cat. no. 9001297) and provided accessories
Note: Required accessories are detailed in the respective extraction kit handbooks.
- QIAAsymphony software version 4.0 or later
- QIAAsymphony Tissue_LC_200_DSP protocol for automated gDNA preparation from FFPE samples (see www.qiagen.com/qiasymphony-dsp-dna-kits)
- QIAAsymphony circDNA_2000_DSP protocol for automated ccfDNA preparation from plasma samples (see www.qiagen.com/qiasymphony-dsp-circulating-dna-kit)

Equipment and materials for qPCR

- Rotor-Gene Q instruments* with fluorescence channels for Cycling Green, Cycling Red, and Cycling Yellow (detection of FAM, CAL Fluor Red 610 and HEX, respectively)
- Rotor-Gene Q software version 2.3 (or later)
- Loading Block 72 × 0.1 mL Tubes, aluminum block (cat. no. 9018901) for manual reaction setup with a single-channel pipette
- 72-Well Rotor (cat. no. 9018903), Locking Ring 72-Well Rotor (cat. no. 9018904), and Rotor Holder (cat. no. 9018908)

Warnings and Precautions

Please be aware that you may be required to consult your local regulations for reporting serious incidents that have occurred in relation to the device to the manufacturer and/or its authorized representative.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety, where you can find, view and print the SDS for each QIAGEN kit and kit component.

- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Discard sample and assay waste according to your local safety procedures.
- For safety information regarding the QIASymphony SP instrument and the Rotor-Gene Q instrument, refer to the user manual supplied with the instrument.

Precautions

Use of EGFR Plus RGQ PCR Kit requires good laboratory practices, including traceability, maintenance of equipment dedicated to molecular biology, and compliance with applicable regulations and relevant standards.

This kit is intended for research use only and not for use in diagnostic procedures. Reagents and instructions supplied in this kit have been tested for optimal performance.

The user should always pay attention to the following:

- The test is for use with FFPE and plasma specimens.
- Use extreme caution to prevent contamination of samples and reagents with *EGFR*-positive material (i.e., Positive Control) or potentially *EGFR*-positive material (i.e., specimens to be tested).
 - Change the scalpels between samples when scraping the tissue.
 - Use separate, dedicated pipettes for DNA extraction/preparation, setting up PCR reaction mixes (pre-PCR reaction mix preparation) and adding DNA template into PCR tubes.
 - Use fresh aerosol-resistant pipette tips for all pipetting steps to avoid cross-contamination of the samples and reagents. Use extreme caution to prevent DNA or PCR product carryover contamination which may result in a false-positive signal.
 - Preparation and dispensing of reaction mixes must be carried out in a dedicated area separate from the DNA preparation area where no DNA matrices (DNA, plasmid, or PCR products) are introduced. In this same area, add water in NTC tubes and close them.
 - Add DNA template in a separate zone, preferably in a separate room, with dedicated equipment (pipettes, tips, etc.).
 - Rotor-Gene Q tubes must not be opened after the PCR run has finished. This is to prevent laboratory contamination with post-PCR products.
- *EGFR* Plus RGQ PCR Kit reagents should be protected from light, temperature, and repeated thawing and freezing, otherwise the kit performance may be altered.
- Frozen components are thawed completely at room temperature (15–25°C) (or in a refrigerator (2–8°C) and protected from light. Check regularly to see whether the material is already thawed.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Reagents for the *EGFR* Plus RGQ PCR Kit have been optimally diluted. Do not dilute reagents further as this may result in a loss of performance.

- Do not use reaction volumes (reaction mix plus sample) of less than 25 μ L since this will increase the risk of a false-negative result.
- All reagents supplied in the EGFR Plus RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same EGFR Plus RGQ PCR Kit.
- Do not substitute the reagents in the EGFR Plus RGQ PCR Kit or between EGFR Plus RGQ PCR Kit lots, as this may affect performance.
- Do not use expired or incorrectly stored components.
- Caution must be observed to ensure correct sample testing and analysis with emphasis on eliminating wrong sample entry, loading error, pipetting error, and placing the PCR strip tubes into the appropriate positions of the 72-well rotor.
- Make sure the samples are handled in a systematic way to ensure correct identification and traceability.
- Use extreme caution to prevent contamination by DNase, which may cause degradation of the template DNA. Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials) and wear gloves when performing the assay.

Note: The product is to be used only by experienced laboratory personnel familiar with laboratory procedures and the Rotor-Gene Q 5plex HRM instrument.

Reagent Storage and Handling

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

Shipping condition

The EGFR Plus RGQ PCR Kit is shipped on dry ice and must still be frozen on arrival. If any component of the EGFR Plus RGQ PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (visit www.qiagen.com).

For shipping conditions relating to the DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

Storage conditions

The EGFR Plus RGQ PCR Kit should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light.

Note: All fluorescently labeled probes in the reaction mix reagents are light sensitive. Protect reaction mix reagents from light to avoid photo bleaching.

Repeated freezing and thawing should be avoided. Reagents should ideally be subjected to a maximum of 4 freeze–thaw cycles.

For storage and handling information relating to the DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

Stability

When stored under the specified storage conditions, the EGFR Plus RGQ PCR Kit is stable until the stated expiration date on the label. Avoid unnecessary freeze–thawing of the contents of the kit.

Once opened, reagents can be stored in their original packaging at -30 to -15°C until the stated expiration date shown on the packaging. The total time prior to run once the PCR reactions are set up should not exceed 24 hours if stored in the refrigerator (2 – 8°C ; this time includes both the PCR setup and storage).

For stability information relating to the DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

Sample Storage and Handling

Sample material is human genomic DNA extracted from FFPE tumor tissue or circulating cell-free DNA (ccfDNA) extracted from K₂ EDTA plasma.

Samples must be transported according to standard pathology methodology to ensure specimen quality.

Note: All samples must be treated as potentially infectious material.

Note: For optimal use of the reagents in the EGFR Plus RGQ PCR Kit, samples should be batched. If samples are tested individually, this will use more reagents and reduce the number of samples that can be tested with the kit.

FFPE samples

Tumor samples are non-homogeneous and data from a tumor sample may not be concordant with other sections from the same tumor. Tumor samples may also contain non-tumor tissue. DNA from non-tumor tissue would not be expected to contain mutations detected by the EGFR Plus RGQ PCR Kit.

To prepare tissue samples for gDNA extraction:

- Standard formalin fixation and paraffin-embedding procedures should be used. Refer to the relevant extraction kit handbook for more details.
- Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides. Use a trained individual (e.g., a pathologist) to assess a Hematoxylin & Eosin (H&E)-stained section to confirm that there is tumor present. The stained sections must not be used for DNA extraction.
- The starting material for gDNA purification is sections of FFPE tissue (ideally freshly cut).

- Store all FFPE blocks and slides at room temperature. FFPE sections mounted on glass slides may be stored at ambient temperature for up to 1 month prior to DNA extraction.

Plasma samples

Use standard laboratory procedures to prepare plasma from K₂ EDTA whole blood samples. Refer to the relevant extraction kit handbook for more details.

If fresh plasma is used for nucleic acid extraction on the same day, store at 2–8°C until further processing. For longer-term storage, keep plasma frozen at –30 to –15°C or –90 to –65°C. It is recommended to use aliquots, to avoid freeze–thawing of plasma samples. Repeated freeze–thawing leads to denaturation and precipitation of proteins, which may result in reduced yields of circulating cell-free nucleic acids.

Genomic DNA and circulating cell-free DNA samples

Genomic DNA extracted from FFPE tissue and circulating cell-free DNA extracted from plasma should be stored at 2–8°C for short-term storage (up to 24 hours) and –30 to –15°C (or –90 to –65°C) if long-term storage is required. Avoid unnecessary freeze–thawing of the extracted gDNA and ccfDNA. Frozen eluates must not be thawed more than three times.

Procedure

Protocol: DNA extraction and preparation

Things to do before starting

- Make sure the operator is trained in using the instruments and extraction kits needed for DNA extraction and sample preparation. If required, instrument training can be provided upon installation (see “Ordering Information”, page 91).
- Read the “Materials Required but Not Provided” section of each extraction kit handbook to identify the accessories required for each procedure:
- QIASymphony DSP DNA Mini Kit (cat. no. 937236) for automated gDNA preparation (from FFPE samples)
- QIASymphony DSP Circulating DNA Kit (cat. no. 937556) for automated ccfDNA preparation (from plasma samples)
- QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual gDNA preparation (from FFPE samples)
- QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual ccfDNA preparation (from plasma samples)

Protocol: gDNA extraction from FFPE samples

The EGFR Plus RGQ PCR Kit has only been tested in combination with QIAGEN Deparaffinization Solution (cat. no. 19093 or 939018) for FFPE section deparaffinization with the following DNA extraction kits:

- QIASymphony DSP DNA Mini Kit (cat. no. 937236) for automated extraction
- QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual extraction

Important points before starting

Applicable to automated extraction and manual extraction protocols:

- Make sure the DNA extraction reagents have not expired and have been transported and stored under the correct conditions.
- Do not use expired or incorrectly stored components.
- Between one and four FFPE tissue sections, each with a thickness of 10 μm , or between two and eight sections with a thickness of up to 5 μm can be combined in one preparation.
- Use only the Deparaffinization Solution for FFPE deparaffinization, according to the procedure, "Pretreatment protocol for use with the QIA Symphony DSP DNA Mini Kit" on page 30 or "Protocol: Pretreatment protocol for use with the QIAamp DSP DNA FFPE Tissue Kit" on page 33.

Note: Deparaffinization Solution is not supplied with extraction kits and should be ordered separately (see "Ordering Information", page 91).

- Use RNase A to minimize RNA content (included in procedure "Protocol: FFPE section deparaffinization with QIAGEN Deparaffinization Solution" on page 30).
Note: RNase A is not supplied with extraction kits and should be ordered separately (see "Ordering Information", page 91).
- Sample dilution may be necessary prior to qPCR testing (see "Protocol: gDNA quantification and normalization", page 38) or for storage.
- DNA isolated from FFPE samples is usually of lower molecular weight than DNA from fresh or frozen samples. The degree of fragmentation depends on the type and age of the sample and the conditions used for fixation.
- For DNA storage after extraction, see "Genomic DNA and circulating cell-free DNA samples", page 26.

Protocol: Automated gDNA extraction from FFPE samples using the QIASymphony SP

If using the QIASymphony DSP DNA Mini Kit (cat. no. 937236) for automated extraction, carry out the DNA extraction according to the instructions in the handbook, noting the following:

- Use only the Deparaffinization Solution for FFPE deparaffinization according to the procedure, “Pretreatment protocol for use with the QIASymphony DSP DNA Mini Kit”, page 30.
Note: Deparaffinization Solution is not supplied with the extraction kits and should be ordered separately (see “Ordering Information”, page 91).
- Select the Tissue_LC_200_V7_DSP protocol on the QIASymphony SP instrument (for more details, refer to the QIASymphony SP Protocol Sheet Tissue_LC_200_V7_DSP protocol)
- Use 50 µL elution volume.
- For any supplementary information concerning the QIASymphony SP instrument, refer to the user manual supplied with the instrument.

Protocol: Manual gDNA extraction from FFPE samples

If using the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual extraction, carry out the DNA extraction according to the instructions in the handbook, noting the following:

- Use only the Deparaffinization Solution for FFPE deparaffinization according to the procedure “Protocol: Pretreatment protocol for use with the QIAamp DSP DNA FFPE Tissue Kit”, page 33.
Note: Deparaffinization Solution is not supplied with the extraction kits and should be ordered separately (see “Ordering Information”, page 91).
- Use 50 µL elution volume.

Protocol: FFPE section deparaffinization with QIAGEN Deparaffinization Solution

Pretreatment protocol for use with the QIASymphony DSP DNA Mini Kit

This pretreatment protocol is for use with the QIASymphony DSP DNA Mini Kit (for automated extraction) and is based on the *QIASymphony SP Protocol Sheet Tissue_LC_200_V7_DSP* protocol (Method 1: deparaffinization using the Deparaffinization Solution).

Important points before starting

- Equilibrate all buffers to room temperature and equilibrate Deparaffinization Solution to 20–25°C.
- QIASymphony magnetic particles co-purify RNA and DNA if both are present in the sample. In order to minimize RNA content in the sample, add RNase A to the sample at the step indicated in the pretreatment protocol below.
- Deparaffinization Solution, RNase A and Buffer ATL are not supplied with the QIASymphony DSP DNA Mini Kit and must be ordered separately (see “Ordering Information”, page 91).

Things to do before starting

- Preheat a thermomixer or shaker-incubator to 56°C for use in step 7.
- Check ATL buffer for white precipitate. If necessary, dissolve precipitate according to the protocol described in *QIASymphony SP Protocol Sheet Tissue_LC_200_V7_DSP protocol*.

Procedure

Starting with FFPE blocks only

1. Using a scalpel, trim excess paraffin off the sample block. Cut between one and four sections of 10 μm thickness or between two and eight sections of 5 μm thickness.
Note: If the sample surface has been exposed to air, discard the first 2–3 sections.
2. Immediately place the section(s) in a 2 mL sample tube compatible with the sample tube carrier of the QIASymphony SP (not supplied; e.g., Sarstedt, cat. no. 72.693).
3. Continue with step 4 below ("For all samples").

Starting with FFPE sections on slides only

1. Put 1 drop of Deparaffinization Solution on each slide using dedicated pipettes for sample preparation.
2. Scrape off the sample material with a single-use, sterile scalpel to collect the entire tissue. Place the aggregates in a 2 mL sample tube compatible with the sample tube carrier of the QIASymphony SP (not supplied; e.g., Sarstedt, cat. no. 72.693).
3. Continue with step 4 below ("For all samples").

For all samples

4. Add 200 μL Buffer ATL to the sections.
5. Add 20 μL proteinase K.
Note: Use proteinase K from the enzyme rack of the QIASymphony DSP DNA Mini Kit.
6. Add 160 μL or 320 μL Deparaffinization Solution (see Table 2) and mix by vortexing.

Table 2. Volume of Deparaffinization Solution required

Section thickness	Number of sections	Volume of Deparaffinization Solution
5 μm	1–4	160 μL
	5–8	320 μL
10 μm	1–2	160 μL
	3–4	320 μL

- Place the tube in a ThermoMixer or shaker-incubator and incubate at 56°C for 1 hour with shaking at 1000 rpm until the tissue is completely lysed.

Note: Lysis time varies depending on the tissue type processed. For most tissues, lysis is completed within 1 hour. If lysis is incomplete after 1 hour as indicated by the presence of insoluble material, lysis time can be prolonged or insoluble material can be pelleted by centrifugation. Overnight lysis is possible and does not affect the preparation.

- Incubate at 90°C for 1 hour.

Note: The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block reaches 90°C.

- To minimize RNA content in the sample, add 2 μL RNase A (100 mg/mL) to the lower phase and incubate for 2 minutes at room temperature before continuing with step 10. Allow the sample to cool to room temperature before adding RNase A.
- Centrifuge at full speed for 1 minute at room temperature.
- Carefully transfer tubes (containing both phases) to the sample carrier of the QIASymphony SP.
- Proceed to extraction following the instructions in the *QIASymphony DSP DNA Mini Kit Handbook* (use 50 μL elution volume).

Protocol: Pretreatment protocol for use with the QIAamp DSP DNA FFPE Tissue Kit

This pretreatment protocol is for use with the QIAamp DSP DNA FFPE Tissue Kit (for manual extraction) and is based on "*QIAGEN Supplementary Protocol: Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution*".

Important points before starting

- Perform all centrifugation steps at room temperature.
- Equilibrate all buffers to room temperature; equilibrate Deparaffinization Solution to 20–25°C.
- Deparaffinization Solution, RNase A and Buffer ATL are not supplied with the QIAamp DSP DNA FFPE Tissue Kit and must be ordered separately (see "Ordering Information", page 91).

Things to do before starting

- Preheat a thermomixer or heated orbital incubator to 56°C for use in steps 6 and 10. If a thermomixer or heated orbital incubator is not available, a heating block or water bath can be used instead.
- If Buffer AL or Buffer ATL contain precipitates, dissolve precipitate according to the protocol described in QIAamp DSP DNA FFPE Tissue Kit. .
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.

Procedure

Starting with FFPE blocks only

1. Using a scalpel, trim excess paraffin off the sample block. Cut into sections 5–10 μm thick.
Note: If the sample surface has been exposed to air, discard the first 2–3 sections.
2. Immediately place the section(s) in a 1.5 mL or 2 mL microcentrifuge tube (not supplied).
3. Continue with step 4 below (“For all samples”).

Starting with FFPE sections on slides only

1. Put 1 drop of Deparaffinization Solution on each slide using dedicated pipettes for sample preparation.
2. Scrape off the sample material with a scalpel to collect the entire tissue. Put the aggregates in a 1.5 mL or 2 mL microcentrifuge tube (not supplied).
3. Continue with step 4 below (“For all samples”).

For all samples

4. Add 160 μL or 320 μL Deparaffinization Solution (Table 3) and vortex vigorously for 10 seconds.

Table 3. Volume of Deparaffinization Solution required

Section thickness	Number of sections	Volume of Deparaffinization Solution
5 μm	1–4	160 μL
	5–8	320 μL
10 μm	1–2	160 μL
	3–4	320 μL

5. Centrifuge briefly to collect the sample in the bottom of the tube.
6. Incubate at 56°C for 3 minutes and allow to cool at room temperature.
7. Add 180 µL Buffer ATL, and mix by vortexing.
8. Centrifuge for 1 minute at 11,000 x *g* (10,000 rpm). Two phases appear (blue and clear).
9. Add 20 µL proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
10. Incubate at 56°C for 1 hour (or until the sample has completely lysed).
11. Incubate at 90°C for 1 hour.

The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA.

Note: If using only one heating block, leave the sample at room temperature after the 56°C incubation in step 10, until the heating block has reached 90°C for step 10.

12. Briefly centrifuge the 1.5 mL tube to remove drops from inside the lid.
13. Transfer the lower, clear phase into a new 2 mL microcentrifuge tube.
14. Add 2 µL RNase A (100 mg/mL) and incubate for 2 minutes at room temperature.
15. Continue with step 12 (addition of Buffer AL) of the *QIAamp DSP DNA FFPE Tissue Kit Handbook* (use 50 µL elution volume).

Protocol: ccfDNA extraction from plasma samples

The EGFR Plus RGQ PCR Kit has been tested in combination with the following DNA extraction kits:

- QIASymphony DSP Circulating DNA Kit (cat. no. 937556) for automated ccfDNA extraction (from plasma samples)
- QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual ccfDNA extraction (from plasma samples)

Important points before starting

Applicable to automated extraction and manual extraction protocols:

- Make sure the DNA extraction reagents have not expired and have been transported and stored under the correct conditions.
- Do not use expired or incorrectly stored components.
- Starting material for ccfDNA purification should be plasma prepared from K2 EDTA whole blood samples. Samples can be either fresh or frozen (provided that they have not been frozen and thawed more than once).
- The concentration of circulating cell-free nucleic acids in biological fluids such as plasma is usually low and varies considerably between individuals. Therefore, ccfDNA extracted from plasma samples will not be quantified nor normalized (no dilution), and is used directly in the qPCR reaction.
- For DNA storage after extraction, refer to the section “Genomic DNA and circulating cell-free DNA samples”, page 26.

Protocol: Automated ccfDNA extraction from plasma samples using the QIAasymphony SP

If using the QIAasymphony DSP Circulating DNA Kit (cat. no. 937556) for automated extraction, carry out the DNA extraction according to the instructions in the handbook, noting the following:

- Select the circDNA_2000_DSP_V1 protocol on the QIAasymphony SP instrument (for protocol details, refer to QIAasymphony SP Protocol Sheet circDNA_2000_DSP_V1)
Important: The recommended sample volume for the circDNA_2000_DSP is 2 mL. However, we recommend starting with 2.4 mL to prevent any extraction failure during initial pipetting, as specified in the “Troubleshooting Guide” of the *QIAasymphony DSP Circulating DNA Kit Handbook*. If insufficient sample is available, add sterile PBS (not supplied) to the sample up to the required sample volume before loading the sample.
- Use a 60 µL elution volume
- For any supplementary information concerning the QIAasymphony SP instrument, refer to the user manual supplied with the instrument.

Protocol: Manual ccfDNA extraction from plasma samples

If using the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual purification, carry out the DNA extraction according to the instructions in the handbook noting the following:

- Purification of circulating nucleic acids is performed from 2 mL of plasma.
- A vacuum manifold (e.g., the QIAvac 24 Plus with the QIAvac Connecting System) and a vacuum pump capable of producing a vacuum of –900 to –800 mbar (e.g., QIAGEN Vacuum Pump) are required for the protocol.
- Use a 60 µL elution volume.

Protocol: gDNA quantification and normalization

Things to do before starting

If using automated extraction procedures, check the “Validity of result” column for each sample on the QIAAsymphony SP result file once the run is finished:

- **Valid status:** Proceed to gDNA quantification.
- **Unclear status:** Can be processed depending on the origin of the flag (for details regarding possible origins of “unclear” flagging, refer to the *QIAAsymphony SP/AS User Manual*).
- **Invalid status:** Sample is rejected. Repeat the extraction step.

Procedure

gDNA extracted from FFPE samples should be quantified.

If measured concentration is less than 4 ng/μL, the sample must be re-extracted with more sections (to a maximum of eight sections of 5 μm or four sections of 10 μm).

If measured concentration is above 6 ng/μL, the sample must be diluted to 5 ng/μL using the Water for sample dilution provided in the EGFR Plus RGQ PCR Kit, according to the formula:

$$C_i \times V_i = C_f \times V_f$$

Where

C_i: Initial concentration of the extracted gDNA

C_f: Final concentration to be targeted = 5 ng/μL

V_f: Final volume needed to perform a EGFR Plus RGQ PCR run (i.e., 20 μL + extra-volume for pipetting error)

V_i : Initial volume of the extracted gDNA to be pipetted and diluted with the Water for sample dilution provided in the EGFR Plus RGQ PCR Kit (Water volume to add = $V_f - V_i$)

Each PCR reaction is optimized for 25 ng gDNA diluted in a final sample volume of 5 μ L. As each sample is tested with the four EGFR reaction mixes, a total of 100 ng per tested sample is needed.

Note: Make sure the correct elution buffer is used to calibrate the quantification instrument.

ccfDNA extracted from plasma samples should not be quantified. Each PCR reaction is optimized for 5 μ L of pure extracted ccfDNA. As each sample is tested with the four EGFR reaction mixes, a total of 20 μ L per tested sample is needed.

Protocol: EGFR mutation assessment by qPCR on Rotor-Gene Q instrument

Important points before starting

- Make sure the operator is trained in using the instruments for qPCR. If required, instrument training can be provided upon installation (see “Ordering Information”, page 91).
- Read “Precautions”, page 20, and become familiar with all components of the kit prior to use.
- The EGFR Plus RGQ PCR Kit must be run on a Rotor-Gene Q 5plex HRM instrument* using the Rotor-Gene Q software version 2.3 or later.
- Take time to familiarize yourself with the Rotor-Gene Q 5plex HRM instrument and the Rotor-Gene software before starting the protocol. See the user manuals for the instrument and the Rotor-Gene Q software for details.
- If you are using automated extraction procedures, check the “Validity of result” column for each sample on the QIA Symphony SP result file once the run is finished, see Protocol: gDNA quantification and normalization, page 38.
- If you are using gDNA extracted from FFPE, the sample should be quantified and diluted to 5 ng/ μ L, see “Protocol: gDNA quantification and normalization”, page 38.
- If you are using ccfDNA extracted from plasma, samples should be used undiluted.

* Ensure that instruments and equipment have been checked and calibrated according to the manufacturer’s recommendations.

Setting up the qPCR

Using the EGFR Plus RGQ PCR Kit, it is recommended to test six DNA samples in the same experiment to optimize use of the controls and reaction mixes. However, up to 16 samples can be tested in the same experiment.

Things to do before starting

- Cool the Loading Block (72 x 0.1 mL tubes) in a refrigerator (2–8°C).
- Before each use, thaw all necessary components.
Note: Do not exceed 1 hour at room temperature for the thawing step to avoid any material degradation. If more time is required, store components at 2–8°C for up to 8 hours.
- Clean the bench area dedicated for the PCR mix preparation to reduce the risk of template or nuclease contamination.
- Vortex the tubes containing controls, Primers and Probes mixes and PCR master mix (3–5 seconds) and then centrifuge briefly before use.

Procedure

1. Prepare the four PCR Reaction Mixes in 1.5 mL or 2 mL tubes (not provided), i.e., mix each Primers and Probes mix (T790M & L861Q Mix, Insertions & G719X Mix, L858R & C797S Mix or Deletions & S768I Mix) with PCR Master Mix, according to the number of samples to be processed.

The volume required for each kit component to make the Reaction Mixes is shown in Table 4. The final PCR reaction volume is 25 μ L after addition of 5 μ L sample DNA or run control template. Extra volume is included to compensate for pipetting variation and to allow preparation of enough reaction mix for planned number of test samples and controls, e.g., six samples plus two controls.

Table 4. Preparation of Reaction Mixes

Component	1 reaction (µL)	8 + 1 reactions (µL)*
EGFR Primers and Probes Mix	7.5	67.5
PCR Master Mix	12.5	112.5
Total volume of Reaction Mix	20	180
Reaction Mix distribution	20 µL per tube	
Test sample distribution	5 µL per tube	
Total volume per reaction	25 µL	

* An extra reaction volume is included to compensate for pipetting error: one extra well for up to 10 wells and two extra wells for up to 20 wells.

2. Return all the EGFR Plus RGQ PCR Kit components to the freezer to avoid any material degradation.
3. Vortex Reaction Mixes for 3–5 seconds and centrifuge briefly.
4. Place the PCR strip tubes on a cooled Loading Block (72 x 0.1 mL tubes) and dispense 20 µL EGFR Reaction Mixes per strip tube following the loading block setup shown in Figure 6.

Note: It is recommended to dispense the 20 µL of the reaction mix by reverse pipetting.

Protocol: Preparing the Rotor-Gene Q 5plex HRM instrument

- Place a 72-well rotor on the Rotor-Gene Q 5plex HRM instrument rotor holder.
- Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Figure 7.

Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions as shown.

- All unused positions should be filled with empty capped strip tubes.

Note: We recommend keeping the four positive controls in positions 1 to 4 and the four no template controls in positions 5 to 8 to facilitate the analysis.

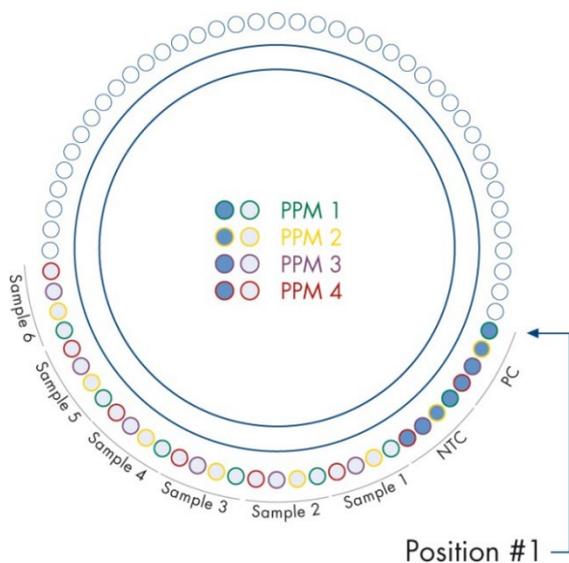


Figure 7. Rotor setup for an experiment with the ThermoFisher EGFR Plus RGQ PCR Kit. From position 1 **PC**: EGFR Positive Control. **NTC**: No template control (water). **PPM 1**: EGFR T790M & L861Q Mix. **PPM 2**: EGFR Insertions & G719X Mix. **PPM 3**: EGFR L858R & C797S Mix. **PPM 4**: EGFR Deletions & S768I Mix. **Sample 1 to Sample 6**: DNA samples. **Note:** All remaining positions ○ should be filled with empty tubes.

11. Attach the locking ring.
12. Load the Rotor-Gene Q 5plex HRM instrument with the rotor and locking ring. Close the instrument lid.

Starting the qPCR run

13. Double-click the **Rotor-Gene Q Software version 2.3 (or later) icon** on the desktop connected to the Rotor-Gene Q instrument to open the software (Figure 8).



Figure 8. Rotor-Gene Q desktop icon.

14. Select **Empty run**, then click **New** (Figure 9).

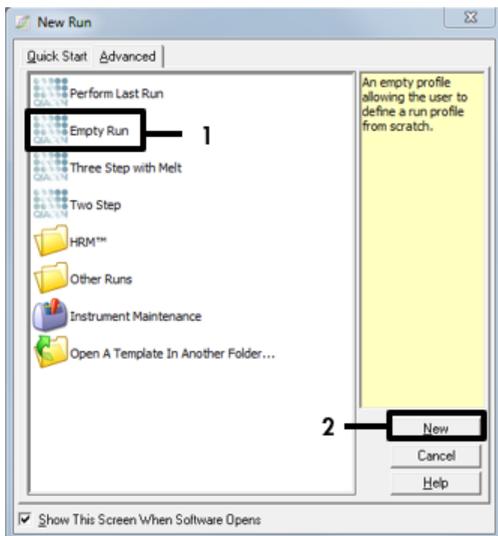


Figure 9. Starting a new run. 1: "Empty run". 2: "New run".

15. In the “New Run Wizard” window, select **72-Well Rotor** from the “Rotor Type” list and check the **Locking Ring Attached** box. Click **Next**.

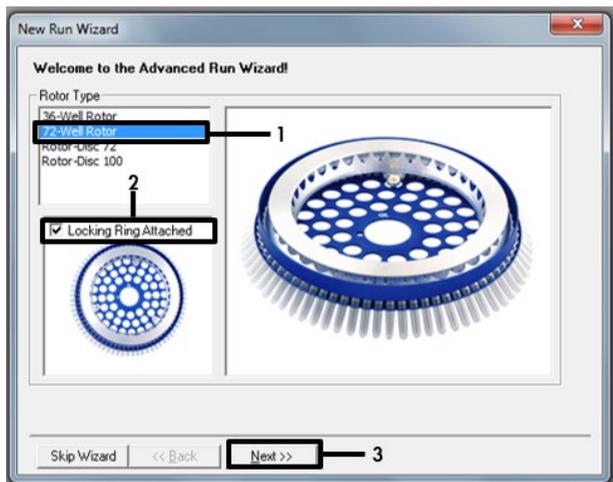


Figure 10. New Run Wizard. 1: “72-Well Rotor”. 2: “Locking Ring Attached”. 3: “Next”.

16. In the “Reaction Volume (µL)” field, set the value to **25**. Ensure that the “Sample Layout” field reads **1,2,3....**Click **Next** (Figure 11). Optionally, enter notes in the “Notes” field.

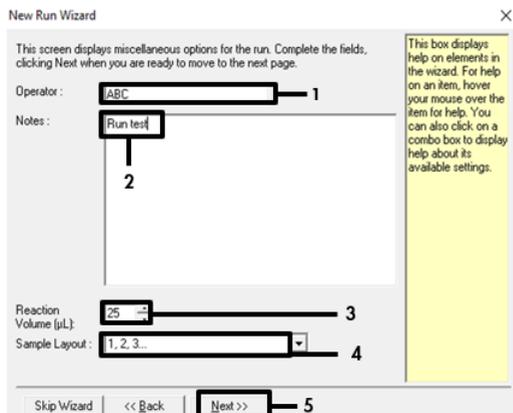


Figure 11. Adding notes. 1: “Operator”. 2: “Notes”. 3: “Reaction volume”. 4: “Sample layout”. 5: “Next”.

17. Click **Edit Profile** (Figure 12).

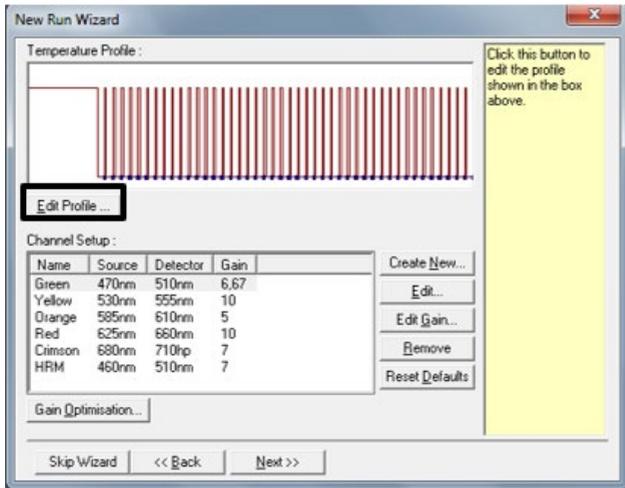


Figure 12. Edit Profile.

18. In the “Edit Profile” window, click **Insert after > New Hold at Temperature** (Figure 13).

Then, set the following values in these fields:

- **Hold Temperature:** Set the value to **95°C**.
- **Hold Time:** Set the value to **10 minutes**.

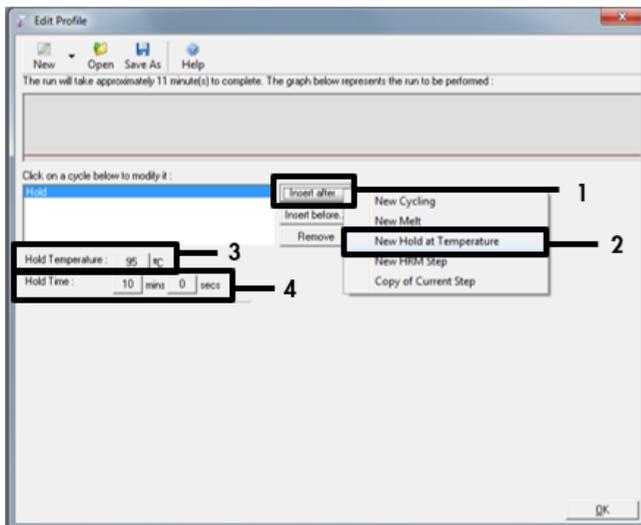


Figure 13. Edit Profile. 1: “Insert after”. 2: “New Hold at Temperature”. 3: “Hold Temperature”. 4: “Hold Time”.

19. Click **Insert after > New Cycling**.

20. In the “This cycle repeats” field, set the value to **45 times**. Remove the automatic third elongation step that was added as default. Then, select the first step and set the value to **95°C for 15 seconds** (Figure 14).

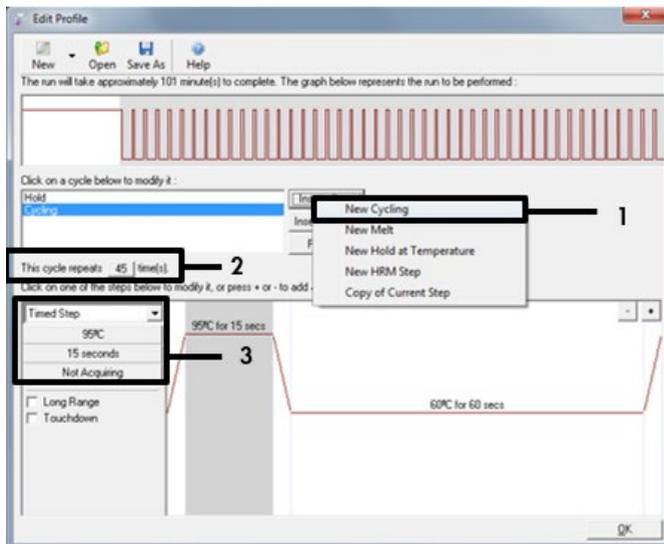


Figure 14. Edit profile. 1: “New cycling”. 2: Cycle repeats 45 times. 3: First step timing.

21. Highlight the second step and set the value to **60°C for 60 sec** (Figure 15).

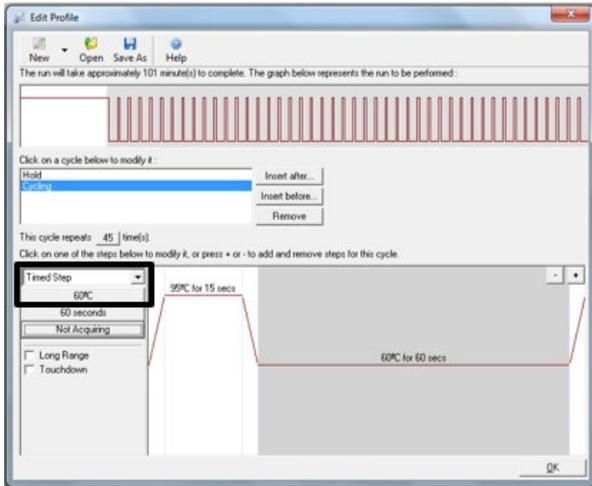


Figure 15. Edit Profile second step. Set the temperature to 60°C.

22. Click **Not Acquiring** to enable data acquisition during this step. In the “Acquisition” dialog box, select **Green, Red, and Yellow** from the “Available channels” list, then click > to move them to the “Acquiring Channels” list. Click **OK** (Figure 16).

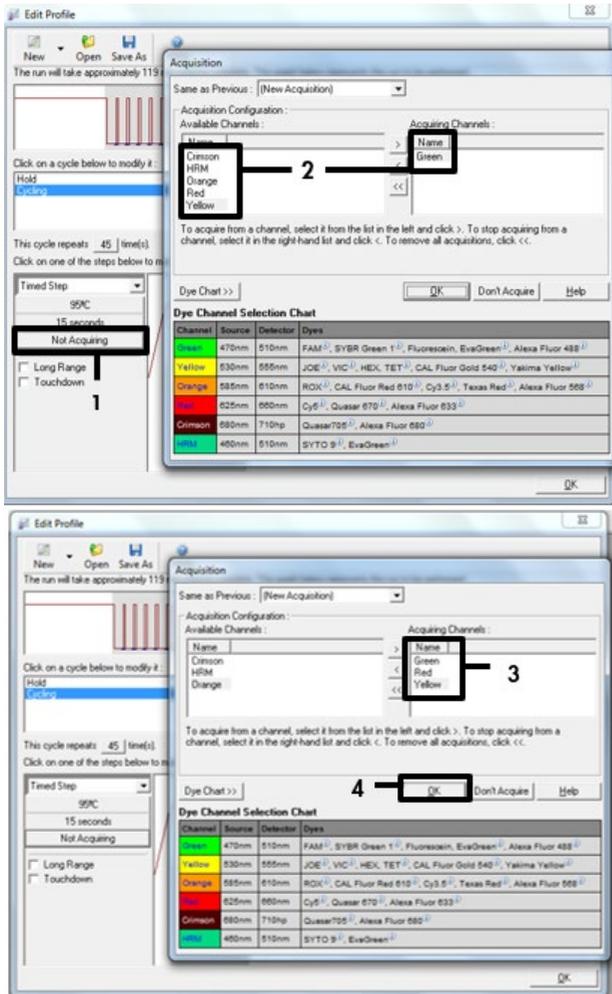


Figure 16. Edit Profile. 1: "Not Acquiring". 2: Selection of acquiring channels. 3: Channels acquired. 4: "OK".

23. Ensure the that acquiring parameter is set to **Acquiring to Cycling A on Green, Red, Yellow** (Figure 17).

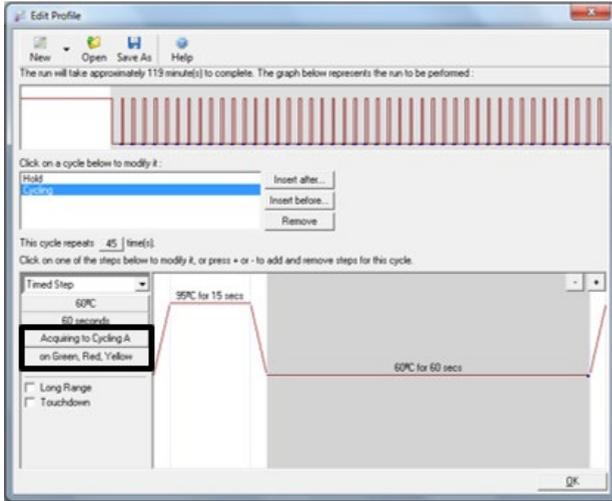


Figure 17. Edit Profile. "Acquiring to Cycling A".

24. In the next window, click Gain Optimisation (Figure 18).

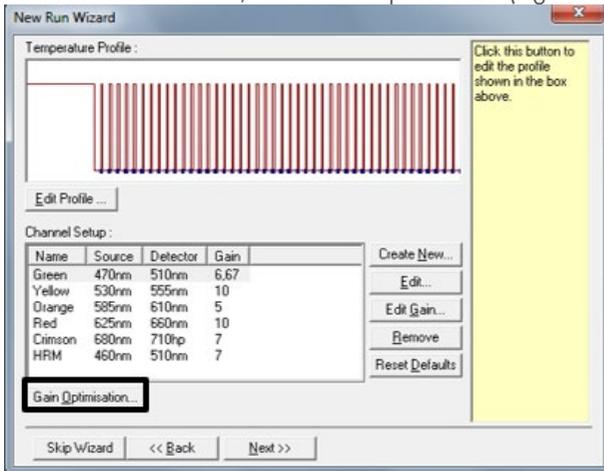


Figure 18. Gain Optimisation.

25. In the “Auto-Gain Optimisation Setup” dialog box, click **Optimise Acquiring** (Figure 19). “Channel settings” are then displayed for each channel. Click **OK** to accept these values for both channels according to the parameters defined in Table 5.

Table 5. Channel settings for the “Optimise Acquiring” step

Parameter	Instruction		
Perform Optimisation before first acquisition	Yes		
Perform Optimisation at 60 degrees at beginning of run	No		
Temperature	60°C for 1 minute		

Channel	Green (FAM Green)	Yellow (HEX)	Red (CAL Fluor Red 610)
Tube position	1	1	1
Min. reading	5	5	5
Max. reading	10	10	10
Gain minimum	-10	-10	-10
Gain maximum	10	10	10

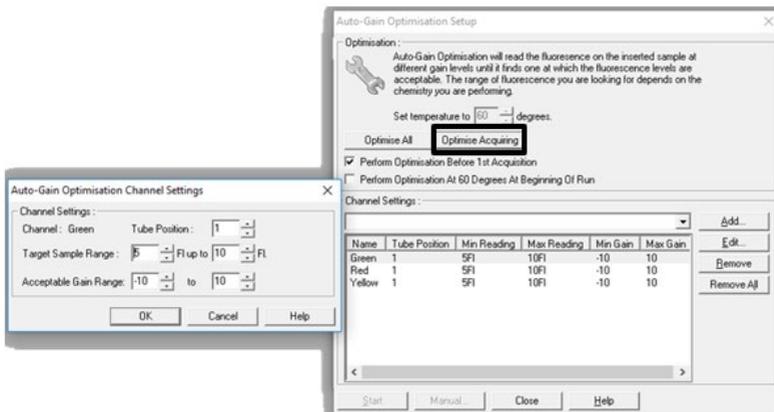


Figure 19. Gain Optimisation settings.

26. Check the **Perform Optimisation before 1st Acquisition** option, then click **Close** to return to the “New Run wizard” window (Figure 20).

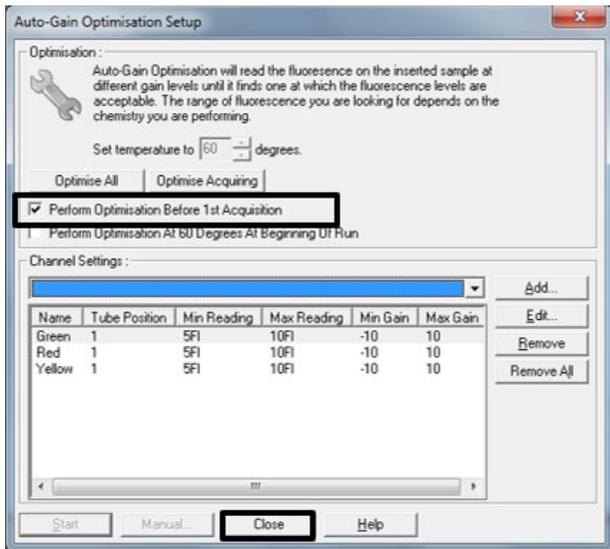


Figure 20. Gain Optimisation. 1: “Perform Optimisation before 1st Acquisition”. 2: “Close”.

27. Click **Next**. To save the template in an appropriate location, click **Save Template**.
28. Review the summary, then click **Start Run** to save the run file and start the run (Figure 21).

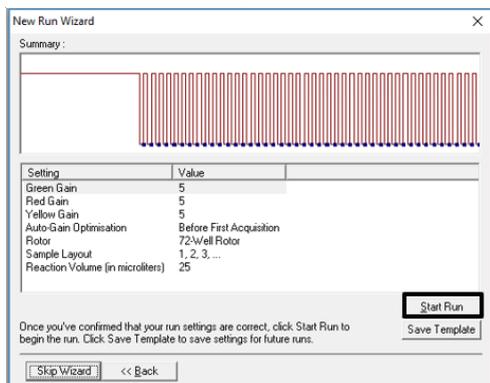


Figure 21. Start run.

29. After the run starts, a new window appears that lets you enter sample names during the run or click **Finish** and enter them at a later time.

Sample Types & Layout

Sample names can be added during the run or after run is complete. Sample type and selected column must be specified to ensure correct analysis parameters are applied. Analysis pages are required for each reaction mix, failure to do so may lead to incorrect results.

1. To define sample layout, click **Samples** to open the "Edit Samples" window (Figure 22).
2. One "Analysis Page" is required for each reaction mix. Click **New** to create a total of four pages.
Note: Navigation between "Pages" can be done using the arrows (Figure 22).
3. "Analysis Pages" should be named to match the reaction mixes: 1) T790M & L861Q Mix; 2) Insertions & G719X Mix; 3) L858R & C797S Mix; 4) Deletions & S768I Mix (Figure 22).

Note: Ensure that **Synchronize Pages** box is unchecked.

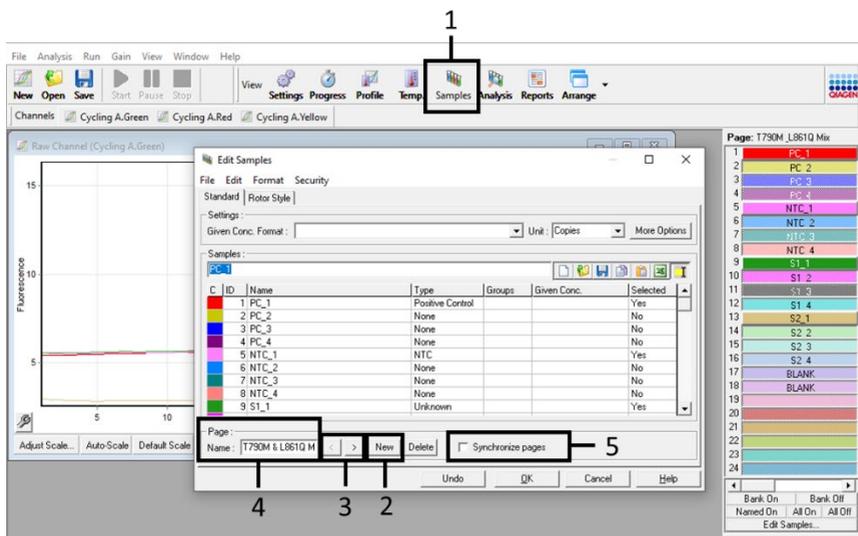


Figure 22. Opening the Edit Sample Window and creating Analysis Pages. 1: Samples button to open edit sample window. 2: New button to create analysis pages. 3: Navigation arrows. 4: Name box for analysis page containing reaction mix name. 5: Synchronize pages box (ensure this is unchecked).

4. On each page, enter the sample names including PC, NTC and samples.

5. Specify sample type according to Table 6 and select **Yes** for the tubes containing the reaction mix relevant for the analysis page (Figure 23).

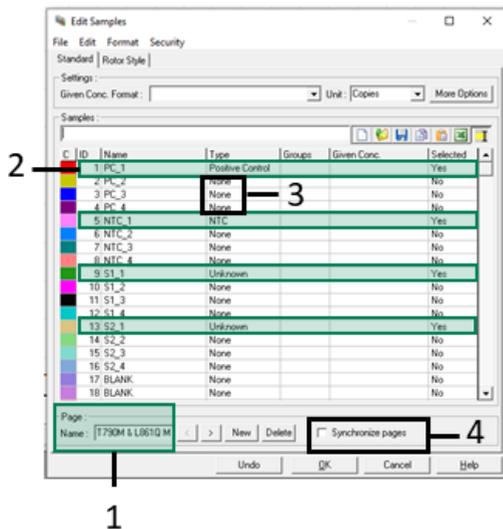
Note: Only tubes containing the relevant reaction mix should be selected on each page (see Figure 6 for sample setup).

6. In tubes not containing the relevant reaction mix, “Sample Type” should be set to **None** and “Selected” should be set to **No** (Figure 23). This ensures only appropriate tubes are analyzed and may lead to incorrect results if performed incorrectly (e.g., on Analysis Page, 1 (T790M & L861Q Mix), the Type for tube 1 should be defined as **PC** and tubes 2–4 should be defined as **None**).

Table 6. Sample types for layout setup in the Rotor-Gene Q

Sample type	Description
None	No sample in that position
NTC	No template control
Negative Control	Negative control
Positive Control	Positive control
Unknown	Unknown sample to be analyzed
Standard	Standard values are used to construct a standard curve to calculate unknown sample concentrations
Calibrator (RQ)	A calibrator is assigned a value of 1 and all other sample concentrations are calculated relative to this sample

Analysis page 1: T790M & L861Q Mix



Analysis page 2: Insertions & G719X Mix

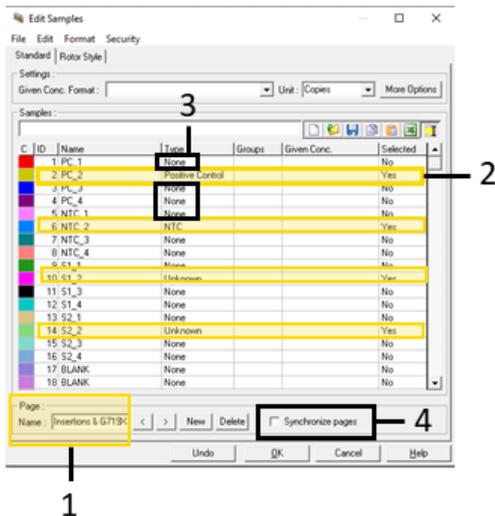


Figure 23. Example of sample layout for analysis pages. 1: Name box for analysis page containing reaction mix name. 2: Type is set for the relevant tubes (Table 7) Selected is set to Yes. 3: Type set to None for tubes not containing named reaction mix. 4: Synchronize pages box is unchecked.

Table 7. Analysis pages, one analysis page should be created for each reaction mix

Reaction Mix Name	Analysis Page No	Tube selected for analysis
T790M & L861Q Mix	1	1 (PC), 5 (NTC), Sample(s) 9, 13, 17, 21,25, 29
Insertions & G719X Mix	2	2 (PC), 6 (NTC), Sample(s) 10,14, 18, 22, 26, 30
L858R & C797S Mix	3	3 (PC), 7 (NTC), Sample(s) 11, 15, 19, 23, 27, 31
Deletions & S768I Mix	4	4 (PC), 8 (NTC), Sample(s) 12, 16, 20, 24, 28, 32
T790M & L861Q Mix	1	1 (PC), 5 (NTC), Sample(s) 9, 13, 17, 21,25, 29

Tubes containing this reaction mix should be selected for analysis and the type should be defined. All other tubes should have type set to None. Failure to do so may invalidate the run.

7. After the run is complete, analyze the data according to “Interpretation of Results” page 60, as appropriate.

Interpretation of Results

After the run is complete, analyze the data as follows.

Software analysis settings

1. Open the appropriate run file using the Rotor-Gene Q series software (version 2.3 or later).
2. If the samples are not named before starting the run, click **Edit Samples**.
3. Insert the sample names in the "Name" column and select the appropriate sample type (as detailed in Sample Types & Layout).

Note: Leave the names of any empty wells blank, select **None** as sample type, and change the "Selected" column to **No**. Incorrect sample type and selection can lead to incorrect analysis parameters being applied.

4. Go to "Analysis" tab and start with **Cycling A. Green** (Figure 23).

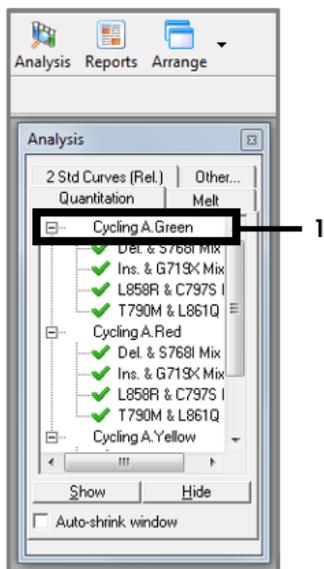


Figure 24. “Analysis” tab for Cycling A. 1: “Cycling A. Green”. Once the correct layout has been used, each of the four reaction mixes will be displayed in the analysis for each channel.

5. Analyze the parameters selection as follows (Figure 24).

- 5a. Click the name of the *EGFR* reaction mix to be analyzed. Check that the **Dynamic Tube** and **Slope Correct** options are selected. Click **Take Off Adjustment** and adjust the cycle to be used as take-off point.

Note: If analysis for individual channels is not present, refer to section “Sample Types” and “Layout” and ensure “Analysis” pages are set up correctly)

- 5b. If take-off point is calculated before cycle 15, use cycle 20 as the take-off point.
- 5c. Click **Outlier Removal**, set the “NTC threshold” field to **10%**, and check that the **Reaction Efficiency Threshold** option is disabled (Figure 25).
- 5d. Click **Linear Scale** and set the threshold to **0.03**.



Figure 25. Setting the Dynamic Tube, Slope Correct, Linear Scale and Threshold. 1: “Dynamic Tube” and “Slope Correct”. 2: “Take Off Adj.” and “Outlier Removal”. 3: “Take Off Point Adjustment”. 4: “Threshold”. 5: “Scale”.

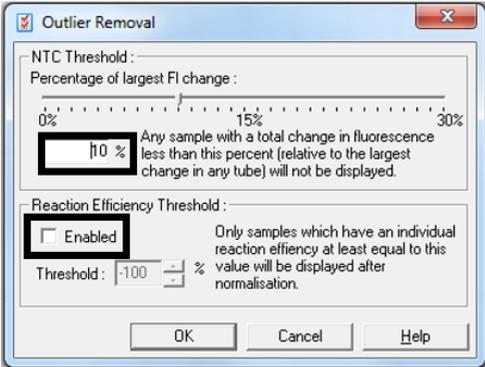


Figure 26. Setting Outlier Removal.

6. Repeat the analysis using the same parameters for **Cycling A. Red** and **Cycling A. Yellow**.
7. For each well, check visually the amplification curves to assess exponential amplification occurred and not an artefact.
8. To interpret the PCR results, click **File > Save As > Excel Analysis Sheet** to export the run report as .csv data.

9. To record all PCR results including the amplification curves, click **Report**. Select the report to record and click **Quantitation (FullReport)** to export as .mht files.
10. Ensure that the raw data (.rex file) have been recorded and proceed to “Data analysis”, page 64.
11. Unload the Rotor-Gene Q instrument and discard the strip tubes according to your local safety regulations.

Data analysis

The data analysis is performed manually following the instruction described in this section.

The PCR cycle at which the fluorescence from a particular reaction crosses a threshold value is defined as the C_T value. The Rotor-Gene Q software interpolates fluorescence signals between any two recorded values. C_T values can therefore be any real number (not limited to integers) within the range 0 to 45.

C_T values for all controls and samples from the 4 *EGFR* assays, i.e., *EGFR* T790M & L861Q Mix, *EGFR* Insertions & G719X Mix, *EGFR* L858R & C797S Mix, *EGFR* Deletions & S768I Mix, in Green (FAM), Red (CFR610), and Yellow (HEX) channels are extracted from the run report (.csv file) or from the single assay reports (.mht file). The .mht files also contain amplification curves of each single assay. The Rotor-Gene Q .rex file is kept as a record of the run raw data.

The following steps are required to determine the *EGFR* mutation status of a sample:

- The qPCR controls of the four *EGFR* assays, i.e., *EGFR* T790M & L861Q Mix, *EGFR* Insertions & G719X Mix, *EGFR* L858R & C797S Mix, *EGFR* Deletions & S768I Mix, must be checked for validity (see “qPCR run validity”, page 65).
- The internal controls of test samples must be checked for validity (see “Validity of test sample”, page 67).
- *EGFR* mutant call for each valid sample is then determined (see “*EGFR* mutant call of test samples”, page 68).
- Semi-quantitative analysis can be performed for the mutations: G719X (A, C or S), S768I, T790M, C797S (a or b), L858R and L861Q (see “Semi-quantitation of ccfDNA using the Rotor-Gene Q”, page 70).

qPCR run validity

For the EGFR qPCR run to be valid, all EGFR assays must be valid, meaning that the C_T values in the Green (FAM), Red (CFR610) and Yellow (HEX) channels for the controls (positive control and no template control) must comply with the run control validity criteria (Table 8).

Table 8. Run control validity criteria

Fluorescence channel	EGFR Reaction Mix	Positive Control (PC) C_T validity range	No template control (NTC) C_T validity criteria	
Green (FAM)	T790M & L861Q	25.74; 32.74	No amplification	
	Insertions & G719X	25.06; 32.06		
	L858R & C797S	23.49; 30.49		
	Deletions & S768I	26.15; 33.98		
Red (CFR ₆₁₀)	T790M & L861Q	21.30; 28.30		
	Insertions & G719X	24.56; 32.38		
	L858R & C797S	22.88; 29.88		
	Deletions & S768I	24.26; 31.86		
Yellow (HEX)	T790M & L861Q	19.89; 26.89		>40.00 or no amplification
	Insertions & G719X			
	L858R & C797S			
	Deletions & S768I			

If one or several run controls are invalid according to the criteria defined above, the run is considered invalid and should be repeated with the 4 *EGFR* Reaction Mixes. If one or several samples are invalid, the samples concerned should be retested with the 4 *EGFR* Reaction Mixes. For additional information related to an invalid result, see the Decision Flowchart presented in Figure 26.

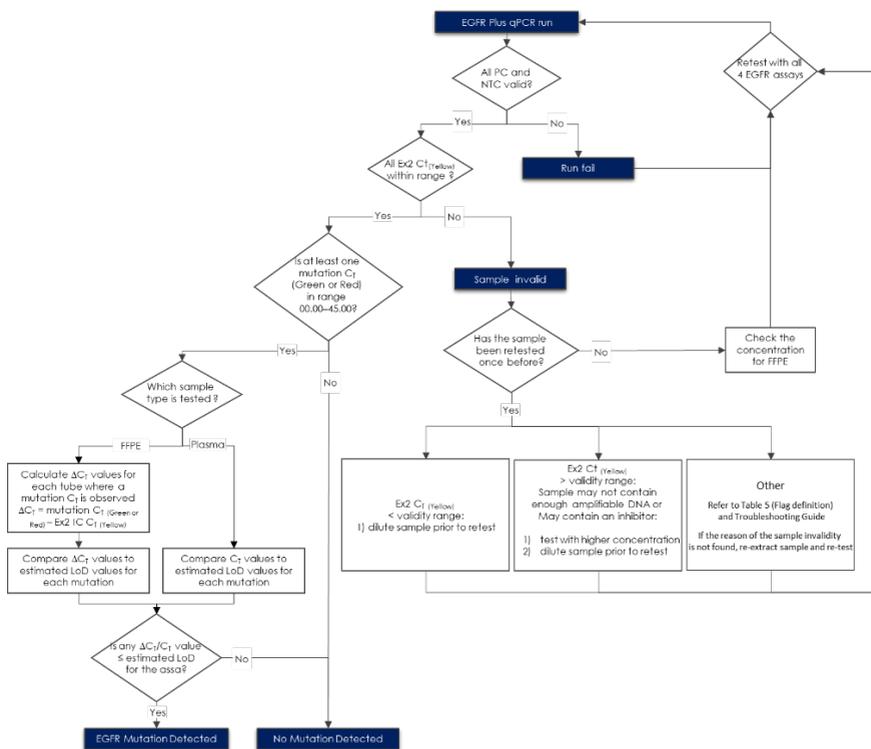


Figure 27. EGFR Plus RQG PCR Kit Decision Flowchart. PC: Positive control. NTC: No template control. Ex2: EGFR exon 2. Ct: Cycle threshold. ΔCt: Delta Ct. MT: Mutant. IC: Internal control. RM: Reaction mix.

If the reason of the sample invalidity is not found:

- Check that the samples have been handled and stored as described in section “Sample Storage and Handling”, page 25.
- Re-extract the FFPE sample with more sections prior to retest.
- Re-extract the FFPE sample by selecting a bigger tumor zone prior to retest.

For other explanations regarding the sample invalidity, please refer to the “Troubleshooting Guide” page 82.

Validity of test sample

If the EGFR qPCR run is valid, then validity of test samples may be assessed.

The internal control (*EGFR* exon 2), assess in Yellow (HEX), must be valid for the sample results to be interpreted. A valid internal control indicates sufficient DNA input and quality as well as the absence of interfering substances.

The internal controls of the test samples are valid, if the C_T values in the Yellow (HEX) channel obtained for test samples are compliant with the validity criteria presented in Table 9 for FFPE samples and Table 10 for plasma samples.

Table 9. Validity criteria for exon 2 Internal Control (IC) in FFPE samples

Fluorescence channel	<i>EGFR</i> reaction mix	FFPE sample C_T validity range
Yellow (HEX)	T790M & L861Q	21.89; 31.10
	Insertions & G719X	
	L858R & C797S	
	Deletions & S768I	

Table 10. Validity criteria for exon 2 Internal Control (IC) in plasma samples

Fluorescence channel	<i>EGFR</i> reaction mix	Plasma sample C_T validity range
Yellow (HEX)	T790M & L861Q	22.10; 31.18
	Insertions & G719X	
	L858R & C797S	
	Deletions & S768I	

If the internal control in a given tube is invalid according to the criteria defined above, the test sample is deemed invalid. In case of invalidity, refer to the Decision Flowchart presented in Figure 26 (page 62).

EGFR mutant call of test samples

If the test sample is valid, the *EGFR* mutant call can be determined.

An *EGFR* mutation presence is assessed based on the delta C_T (ΔC_T) between the target amplification in Green (FAM) or Red (CFR610) channel and the internal control amplification in Yellow (HEX) channel for FFPE samples. Calculate the ΔC_T value as follows:

- ΔC_T for Green target = Green (FAM) C_T – Yellow (HEX) C_T
- ΔC_T for Red target = Red (CFR610) C_T – Yellow (HEX) C_T

An *EGFR* mutation presence is assessed based on the C_T of the target amplification in Green (FAM) or Red (CFR610) channel for plasma samples.

Compare the ΔC_T or C_T value of each test sample in each channel and each *EGFR* reaction mix (8 in total) with the estimated limit of detection of each target. See Table 11 for the FFPE samples and Table 12 for the plasma samples. If the ΔC_T or C_T value is lower or equal to the estimated limit of detection, the respective target is detected.

Table 11. Estimated limit of detection of each EGFR target in FFPE samples

Fluorescence channel	EGFR reaction mix	Target	Estimated limit of detection (ΔC_T)
Green (FAM)	T790M & L861Q	T790M	10.52
	Insertions & G719X	Insertions	9.48
	L858R & C797S	L858R	9.18
	Deletions & S768I	Deletions	10.49
Red (CFR ₆₁₀)	T790M & L861Q	L861Q	9.49
	Insertions & G719X	G719X	8.04
	L858R & C797S	C797S	9.09
	Deletions & S768I	S768I	12.50

Table 12. Estimated limit of detection of each EGFR target in plasma samples

Fluorescence channel	EGFR reaction mix	Target	Estimated limit of detection (C_T)
Green (FAM)	T790M & L861Q	T790M	38.53
	Insertions & G719X	Insertions	36.59
	L858R & C797S	L858R	36.72
	Deletions & S768I	Deletions	40.00
Red (CFR ₆₁₀)	T790M & L861Q	L861Q	34.81
	Insertions & G719X	G719X	40.00
	L858R & C797S	C797S	34.96
	Deletions & S768I	S768I	40.00

The status of each test sample will be one of the following:

- EGFR Mutation Detected
- Mutation Not Detected
- Invalid

Semi-quantitation of ccfDNA using the Rotor-Gene Q

In addition to *EGFR* mutation detection, semi-quantitative analysis can be performed on ccfDNA extracted from Plasma samples for the mutations: G719X (A, C or S), S768I, T790M, C797S (a or b), L858R, and L861Q.

Important point before starting

- If you are performing semi-quantification of ccfDNA, it is required to run duplicates of the DNA standard dilutions for each assay and on each reaction plate.
- Standards must be defined before a standard curve can be created.
- Make sure that a plate layout has been created for each mix and reaction channel (e.g.: page 1 of the "Sample Editing" window for quantitative analysis is "T790M&L861Q" while three pages will be created for semi-quantitative analysis)

Procedure

1. Prepare fresh serial dilutions of the external standards (e.g., *EGFR* DNA Reference Standard can be provided by Horizon Discovery).
2. Vortex for at least 5 seconds, then centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipette tip for each dilution.
Note: Take care not to introduce cross-contamination.
3. Thaw the template nucleic acids.
4. Follow steps 1–12 of "Protocol: EGFR mutation assessment by qPCR on Rotor-Gene Q instrument", page 40.
5. Ensure that the reaction Master Mix and template are thoroughly mixed.
6. It is required to run duplicates of the DNA standard dilutions for each assay and on each reaction plate.

- Continue with steps 13–28 of “Protocol: EGFR mutation assessment by qPCR on Rotor-Gene Q instrument”, page 40.
- Proceed with data analysis.

An example data analysis for semi-quantitation in T790M & L861Q Mix can be found in the next section.

Data analysis for semi-quantification of ccfDNA using Rotor-Gene Q

- Open the Run file using the Rotor-Gene Q software. Go to **File > Open > Browse** to locate the saved file.
- Standards must be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 12.
- Click the **Samples** to open the “Edit Samples” window (Figure 27).



Figure 28. “Edit Samples” tool in the Rotor-Gene Q software.

- Instead of one plate layout per mix (e.g., Page 1/T790M & L861Q Mix, Page 2 Insertions & G719X etc.), one plate layout per assay must be defined (e.g., Page 1/T790M, Page 2/L861Q, Page 3/T790M & L861Q_IC).
- On Page 1/T790M of the “Edit Samples” window, select the sample type from the “Samples” list for each of the wells, e.g., **Standard** for T790M DNA standards, **NTC** for no template controls, **Positive Control** for EGFR Positive Control, **Unknown** for tested samples, and **None** for all empty wells and unanalyzed wells in this channel. In addition, change the “Selected column” to **No** for all empty wells and unanalyzed wells in this channel (Figure 28).

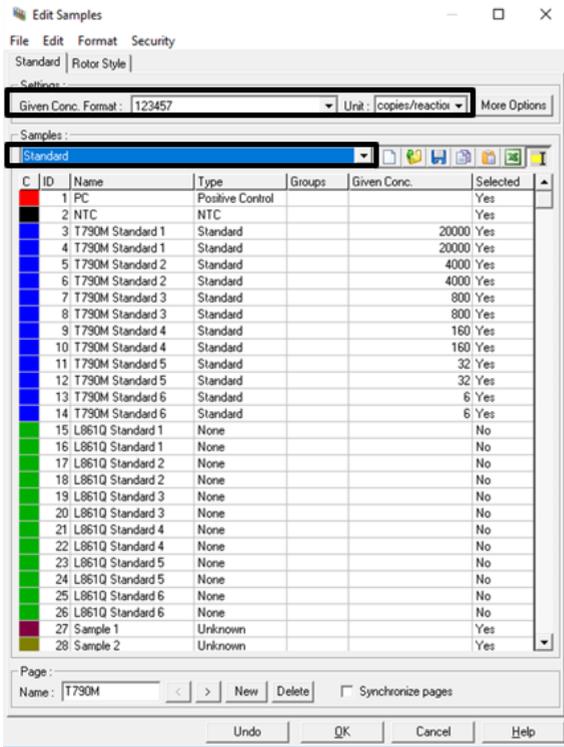


Figure 29. Editing the samples and units in the Rotor-Gene Q software.

- In the "Given Conc." column, enter the DNA concentration of the T790M standards dilutions and define the unit (copies/reaction) using the drop-down list. Enter the sample name (e.g., Standard 1 20000 copies/reaction) (Figure 29).
- Click **New** twice to create two new pages, then check the **Synchronize pages** box (Figure 29).

Important: If you do not check the **Synchronize pages** box, the names of the wells will not be adopted for all channels.

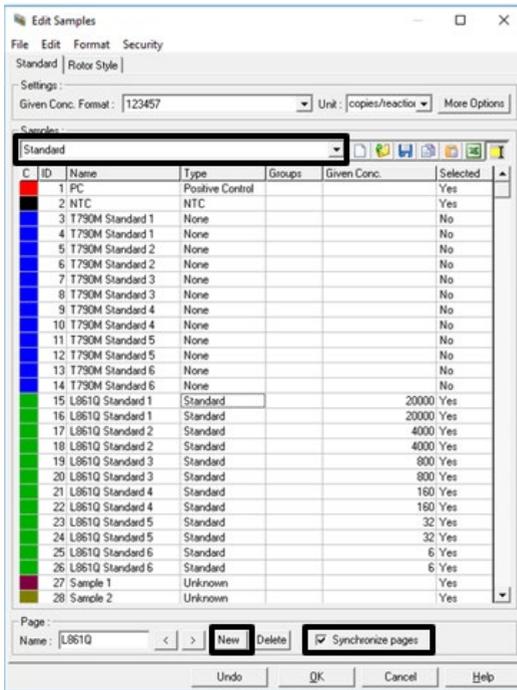


Figure 30. Create a new sample page.

16. On Page 2/L861Q of the Edit Samples window, clear the **Synchronize pages** box. Select **None** as sample type for T790M DNA standards (Figure 29). In the “Given Conc.” column, enter the L861Q DNA standard concentrations and define the unit (copies/reaction) from the drop-down list (Figure 29).

Important: If you do not clear the **Synchronize pages** box, the same concentrations will be adopted for both T790M and L861Q standards, leading to an incorrect calculation of the unknown concentrations.

17. On Page 3/T790M & L861Q_IC of the “Edit Samples” window, leave all DNA standards as **Unknown** in the “Type” column.

18. Click **More Options > Define Suitabilities**. Select the **Only display this sample page when analyzing data** option (Figure 30).

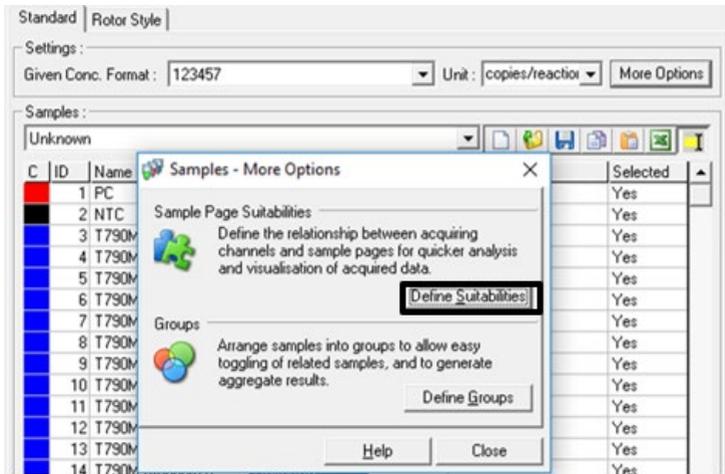


Figure 31. Sample Page Suitabilities.

19. Select the **green channel** for page 1/T790M, **red channel** for page 2/L861Q, and **yellow channel** for page 3/IC. Click **Save and Close**. Click **Close** to close the “Sample Page Suitabilities” dialog box (Figure 31).

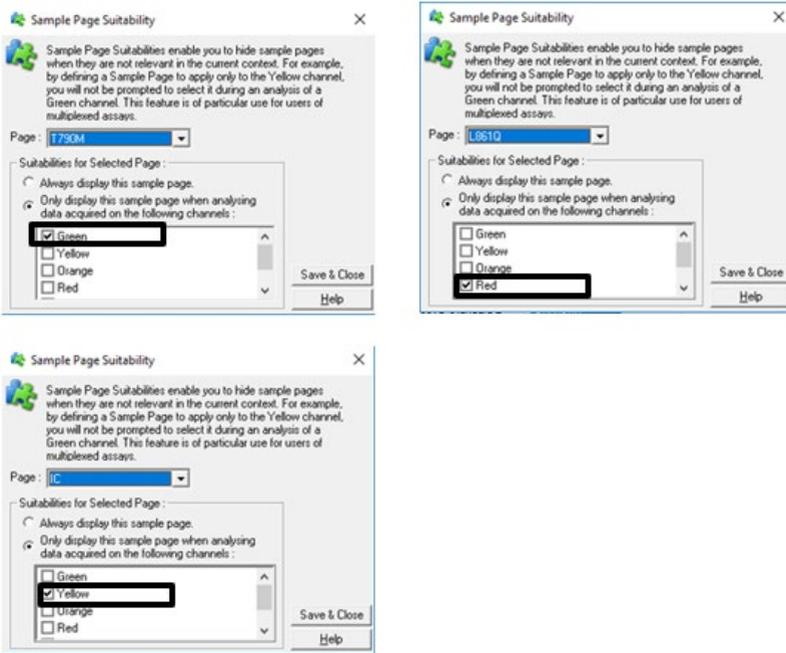


Figure 32. Selecting color channels.

20. Click **OK** in the “Edit Samples” dialog box.
21. To analyze the samples, click **Analysis**. (Figure 32).

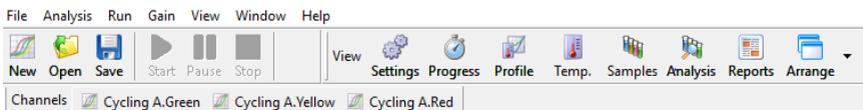


Figure 33. Sample analysis in the Rotor-Gene Q software.

22. On the “Quantitation tab”, select **Page 1/T790M** for the green channel, and click **Show**. Select **Page 2/L861Q** for the red channel, and **Page 3/IC** for the yellow channel, and click **Show**. If the “Autofind Threshold” window opens automatically, click **Cancel**.

The amplification plots for both the green and yellow channels are displayed in the “Quantitation Analysis” dialog box (Figure 33).

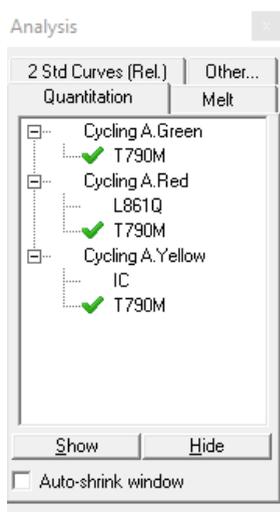


Figure 34. Quantitation tab in the Analysis tool.

23. Select the **Dynamic Tube** and **Slope Correct** options for each channel (Figure 24, page 62).
24. Click **Take Off Adj.** and adjust the cycle to be used as take off point. If take off point was calculated before cycle 15, use cycle 20 as the take-off point (Figure 24, page 62).
25. Click **Outlier Removal**, set the value in the “NTC threshold” field to **10%**, and ensure that **Reaction Efficiency Threshold** is disabled (Figure 25, page 62).
26. Click **Linear scale** and set the threshold to **0.03** (Figure 24, page 62). The C_T values are reported in the “Quantitation Results” window.
27. The Standard Curves are shown in the “Standard Curve” windows for the green and the red channels. View the calculated regression line, slope (M), y-intercept (B), and R^2 values (Figure 34).

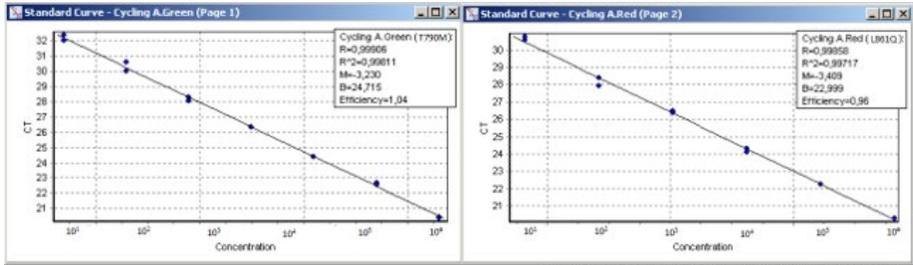


Figure 35. Standard curves in the Rotor-Gene Q software.

28. View the concentration of the unknown samples.

The “Quantitation Results — Cycling A. Green” and the “Quantitation Results — Cycling A. Red” windows display data for selected wells, and respectively summarize the quantity of T790M and L861Q DNA present in the unknown samples. The unit is shown at the top of the column.

29. To interpret the results, go to **File > Save as > Excel Analysis Sheet**. The results will be saved in *.csv format. To export a full report, go to **File > Reports > Quantitation**.

Interpretation considerations

General considerations for data analysis

Real-time PCR data are produced as sigmoidal-shaped amplification plots (when using a linear scale), in which fluorescence is plotted against the number of cycles.

The threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence.

The optimal threshold setting depends on the reaction chemistries used for PCR. Therefore, an optimal threshold setting established for another kit may not be suitable for the EGFR Plus RGQ PCR Kit and may need to be adjusted.

For DNA quantification using the EGFR Plus RGQ PCR Kit, the analysis settings must be adjusted for both reporter dyes.

Standard curve

The standard curve is the best fit for a linear regression to the standard dilution series data. The equation is in the form: $y = mx + b$ (where $x = \log$ concentration and $y = C_T$).

Typically, the R^2 ranges should be as close as possible to 1. If the values differs significantly from 1, see the "Troubleshooting Guide", page 82, for more information.

Slope

The slope (m) describes the PCR efficiency. A slope of -3.3 indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). Typically, the slope ranges between -3.0 to -3.6 . If the values fall outside of this range, see the "Troubleshooting Guide", page 82, for more information.

Y-intercept

The Y-intercept (b) indicates the expected C_T value for a sample with Quantity = 1 (for example, 1 copy/reaction).

Internal control

To analyze the internal control results, see “qPCR run validity” (page 65) and “Validity of test sample” (page 67).

Semi-quantification of unknown samples

The *EGFR* target quantification is automatically performed by the Rotor-Gene Q software. The EGFR Plus RGQ PCR Kit can quantify ccfDNA in 5 μ L plasma samples.

For ccfDNA, C_T values (from Green or Red channels) are plotted against a standard curve obtained from linear regression of standards to calculate each *EGFR* target copies per reaction present in the sample.

Limitations

This product is to be used for research use only, not to be used for diagnosis. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

The product is to be used by laboratory professionals trained in molecular biology procedures and trained in the use of the QIAasymphony SP system and Rotor-Gene Q instrument.

The product is intended for use only on a Rotor-Gene Q real-time PCR cycler, 5plex HRM series.

We recommend the use of the Deparaffinization Solution (including RNase A treatment), QIAamp DSP DNA FFPE Tissue Kit, QIAamp DSP Circulating Nucleic Acid Kit, QIAasymphony DSP DNA Mini Kit, and QIAasymphony DSP Circulating DNA Kit, or the technically equivalent non-DSP kits.

Strict compliance with the *EGFR Plus RGQ PCR Kit Instructions for Use* is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended and will result in a loss of performance. All reagents supplied in the EGFR Plus RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same kit. This may affect performance.

It is important that the amount of gDNA from the FFPE sample is assessed prior to performing sample analysis using the EGFR Plus RGQ PCR Kit. The extraction procedure should be repeated if the gDNA quantity is not sufficient for mutation analysis. gDNA should be diluted if the concentration is too high for mutation analysis.

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

References

1. Pao, W. and Miller, V.A. (2005) Epidermal growth factor receptor mutations, small molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J. Clin. Oncol.* **23**, 2556.
2. Newton, C.R., Graham, A., Heptinstall, L.E., et al. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS) *Nucleic Acids Res.* **17**, 2503.
3. Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., Little, S. (1999). Detection of PCR products using self-probing amplicons and fluorescence. *Nature Biotech.* **17**, 804.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit www.qiagen.com).

Comments and suggestions

Run invalid due to invalid Positive Control

- | | |
|---|---|
| a) One component of the reaction mix not added | Check that the reaction mix has been prepared correctly.
Check that all components of the qPCR reaction mix have been added.
Repeat the qPCR run. |
| b) Reaction mix degraded | Kit has been frozen and thawed too many times, the kit contents were not stored at -30 to -15°C or the primer and probe mixes were not protected from light.
Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit.
Repeat the qPCR run. |
| c) Too low volume pipetted: pipetting volume may be incorrect | Check pipetting scheme and setup of the reaction. Check 5 μL control was added.
Check and recalibrate the pipettes before repeating the qPCR run. |
| d) Fault with Rotor-Gene Q instrument | Check instrument maintenance logs.
Repeat the qPCR run. |
| e) Error in Rotor-Gene Q instrument accessories | The 72-Well Rotor may be incorrectly locked.
Repeat the qPCR run. |
| f) Strip tube and/or sample ID inversion | Check the pipetting scheme and setup of the reaction.
Repeat the qPCR run. |

Comments and suggestions

g) Controls missing or loaded in an incorrect position	Make sure that the correct control is loaded in the correct position. Repeat the qPCR run.
h) Insufficient mixing of control samples	The thawing of the controls was incomplete before loading or the mixing of controls with the reaction mix (pipetting up and down) was not performed correctly. Repeat the qPCR run.
i) Inefficient tube closing	The tube was not efficiently capped, leading to evaporation during the qPCR run. Repeat the qPCR run.
j) The storage conditions for one or more kit components did not comply with the instructions given in "Storage conditions" (page 23)	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
k) The EGFR Plus RGQ PCR Kit has expired	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
l) Wrong software analysis settings	Check software analysis settings. Repeat the qPCR run analysis.
m) Wrong qPCR program settings	Check qPCR program settings. Repeat the qPCR run
n) Incorrect amplification curve (artefact)	Check the corresponding curve for unusual curves (i.e., a straight line). Repeat the qPCR run.

Run invalid due to amplification in No Template Control

a) Cross-contamination or contamination of reagents	Always handle samples, kit components and consumables in accordance with recommended practices to prevent carryover contamination. Make sure tips are changed between pipetting different reagents or when loading different tubes. Prepare the qPCR reaction mix with dedicated material (pipettes, tips, etc.). Prepare the qPCR reaction mix and NTC reaction in a dedicated area where no DNA matrices (DNA, plasmid or PCR products) are introduced. If cross contamination is identified, replace all reagents. Repeat the qPCR run.
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Comments and suggestions

b) Incorrect amplification curve (artefact)	Check the corresponding curve for unusual curves (i.e., a straight line). Repeat the qPCR run.
c) Strip tube and/or sample ID inversion	Check the pipetting scheme and setup of the reaction. Repeat the qPCR run.
d) Wrong software analysis settings	Check software analysis settings. Repeat the qPCR run analysis.

Sample invalid due to no or low amplification in Internal Control

a) Too low sample DNA concentration	The EGFR Plus RGQ PCR Kit is optimized for a 5 ng/ μ L working concentration (DNA from FFPE) and 5 μ L pure extract of ccfDNA from plasma samples. Check the concentration (for FFPE). Repeat the qPCR step for the sample.
b) Poor DNA sample quality	Check the concentration (for FFPE). Repeat the qPCR step with an alternative sample (ideally freshly cut). Repeat the run using a higher sample concentration. Note: Testing higher concentration of gDNA may enable an earlier exon 2 C_T , but will increase the risk of inhibition. Therefore, the C_T exon 2 may be later than with a lower concentration.
c) Presence of inhibitor in the sample	Dilute the sample and repeat the run with lower concentration of sample. Note: Testing a lower concentration may give an earlier exon 2 signal if inhibitors were present in the sample at 5 ng/ μ L. Re-extract the sample (FFPE) with more sections and repeat the run. Note: By eluting more sections in the same volume, the inhibitor concentration may be increased when the sample is normalized at 5 ng/ μ L.
d) Too low volume pipetted: pipetting volume may be incorrect	Check pipetting scheme and setup of the reaction. Check 5 μ L of sample was added. Check and recalibrate the pipettes before repeating the qPCR run.
e) Inefficient tube closing	The tube was not efficiently capped, leading to evaporation during the qPCR run. Repeat the qPCR step for the sample.

Comments and suggestions

f) The storage conditions for one or more kit components did not comply with the instructions given in "Storage conditions" (page 23)	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
g) The EGFR Plus RGQ PCR Kit has expired	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
h) Wrong software analysis settings	Check software analysis settings. Repeat the qPCR run analysis.
i) Wrong qPCR program settings	Check qPCR program settings. Repeat the qPCR run.
j) Abnormal fluorescence	Do not write on the tubes. Use caution when handling tubes. Wear gloves. Check visually the PCR reaction tube for the presence of black magnetic particles (originating from automated QIA Symphony extraction). Particles may be removed as described in the respective extraction kit handbooks.

Sample invalid due to early amplification of the Internal Control in samples

a) Too high sample DNA concentration	The EGFR Plus RGQ PCR Kit is optimized for a 5 ng/ μ L working concentration (FFPE). For gDNA from FFPE: Check DNA concentration. If DNA is not at this concentration, dilute DNA. For ccfDNA from plasma: Dilute the sample. Repeat the qPCR step for the sample.
b) Too high volume pipetted: Pipetting volume may be incorrect	Check pipetting scheme and setup of the reaction. Check 5 μ L sample was added. Check and recalibrate the pipettes before repeating the qPCR run for the sample.
c) The amplification curve may be incorrect	Check the corresponding curve for false positives (i.e., a straight curve instead of exponential amplification). Repeat the qPCR step for the sample.
d) Abnormal fluorescence	Do not write on the tubes. Use caution when handling tubes. Wear gloves. Check visually the PCR reaction tube for the presence of black magnetic particles (originating from automated QIA Symphony extraction). Particles may be removed as described in the respective extraction kit handbooks.

Comments and suggestions

Frequent failure due to high background amplification below the estimated limit of detection (ΔCT)

Fault with Rotor-Gene Q instrument	Check instrument maintenance logs. For example, lens misalignment may lead to higher background. If lens alignment is not part of your maintenance plan, please contact QIAGEN Technical Services for more information and potential intervention.
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Run failure due to inconsistent fluorescence signal in controls and/or samples (over all tubes)

a) Fault with Rotor-Gene Q instrument accessories	Check instrument maintenance logs. Faulty rotor disc.
b) Abnormal fluorescence	Do not write on the tubes. Use caution when handling tubes. Wear gloves. Check visually the PCR reaction tube for the presence of black magnetic particles (originating from automated QIA Symphony extraction). Particles may be removed as described in the respective extraction kit handbooks.

No linearity in ratio of C_T value/crossing point to log of the template amount

Amount of template in unknown sample too high	Linearity is guaranteed within the range of the standard curve. When signals are appearing at very early C_T values, dilute the sample and repeat the reaction.
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Slope of the standard curve used for the semi quantification is not between -3.0 and -3.6 or R^2 value differs significantly from 1

a) Too high/low volume pipetted: pipetting volume may be incorrect	Check pipetting scheme and setup of the reaction. Check 5 μL of each serial dilution point was added. Check and recalibrate the pipettes before repeating the qPCR run.
b) Problem with dilution of standards or wrong serial dilution of the reference material	Ensure that the DNA standard is completely thawed and mixed thoroughly before use. Ensure dilutions of the DNA standard are mixed thoroughly before removing each aliquot for the serial dilution. Do not use a sample volume other than 5 μL for plasma and for FFPE (5 $\text{ng}/\mu\text{L}$). Verify all calculations, and repeat dilution of the DNA standard. Change pipet tips between each dilution step. Repeat the serial dilution and the qPCR run.

Comments and suggestions

c) Run is invalid	Check the run validity. Check the Control (PC and NTC) validity.
d) The EGFR Plus RGQ PCR Kit is expired	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
e) The amplification curve may be incorrect	Check the corresponding curve (i.e., a straight curve instead of exponential amplification). Repeat the qPCR step.
f) Wrong standard identification and/or concentration entered in the Rotor-Gene Q software	Check that Standards are identified as Standards. Check that the correct concentration is assigned to each standard. Repeat the qPCR analysis.
g) Contamination of real-time cyclers	Decontaminate the real-time cycler according to the manufacturer's instructions. Repeat the experiment.
h) Fault with Rotor-Gene Q instrument	Check instrument maintenance logs. Repeat the qPCR step.
i) Wavy curve at high template amounts for highly concentrated targets	In the analysis settings, reduce the number of cycles used for background calculation or reduce the amount of template.
j) Abnormal fluorescence	Do not write on the tubes. Use caution when handling tubes. Wear gloves.
k) Statistical variation	Some variation in the reaction is normal, particularly when the DNA target is present at low copy number. Perform at least duplicates for the standard curve to minimize the effect of this variation. Remove the lowest concentrated dilution of the DNA standard from the standard curve by changing the sample type to "Unknown".

For troubleshooting information relating to DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

For troubleshooting information relating to the QIA Symphony SP instrument and Rotor-Gene Q instrument, refer to the respective user manuals.

Symbols

The following symbols may appear in the instructions for use or on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Material number (i.e., component labeling)
	Components
	Contains
	Number
	Global Trade Item Number
	Temperature limitation
	Manufacturer

Symbol

Symbol definition



Consult instructions for use



Keep away from sunlight



Warning/caution

Contact Information

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Ordering Information

Product	Contents	Cat. no.
EGFR Plus RGQ PCR Kit (24)	For 24 reactions: T790M & L861Q Mix, Insertions & G719X Mix, L858R & C797S Mix, Deletions & S768I Mix, PCR Master Mix, <i>EGFR</i> Positive Control, RNase/DNase-free Water	874601
Rotor-Gene Q and accessories		
Rotor-Gene Q 5plex HRM System	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (Green, Yellow, Orange, Red, Crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training included	9001650
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (Green, Yellow, Orange, Red, Crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9001580
Loading Block 72 x 0.1 mL Tubes	Aluminum block for manual reaction setup with a single-channel pipette in 72 x 0.1 mL tubes	9018901
72-Well Rotor	For holding Strip Tubes and Caps 0.1 ml; requires Locking Ring 72-Well Rotor	9018903

Product	Contents	Cat. no.
Locking Ring 72-Well Rotor	For locking Strip Tubes and Caps, 0.1 mL in the 72-Well Rotor	9018904
Rotor Holder	Metal free-standing holder for assembling tubes and Rotor-Discs® into rotors	9018908
Strip Tubes and Caps, 0.1 mL (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 mL (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
QIASymphony SP		
QIASymphony SP System	QIASymphony sample prep module: includes installation and training, 1 year warranty on parts and labor	9001751
QIASymphony SP	QIASymphony sample prep module: includes 1-year warranty on parts and labor	9001297
Related products		
Deparaffinization Solution (16 mL)	2 x 8 mL Deparaffinization Solution	19093
Deparaffinization Solution (50 mL)	1 x 50 mL Deparaffinization Solution	939018
QIAamp DSP DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers and Collection Tubes (2 mL)	60404
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Buffers and Collection Tubes (2 mL)	56404

Product	Contents	Cat. no.
QIAamp DSP Circulating NA Kit (50)	For 50 preps: QIAamp Mini columns, Buffers, Carrier DNA, QIAGEN Proteinase K and tubes	61504
QIAamp Circulating NA Kit (50)	For 50 preps: QIAamp Mini Columns, Tube Extenders (20 mL), QIAGEN Proteinase K, Carrier RNA, Buffers, VacConnectors, and Collection Tubes (1.5 mL and 2 mL)	55114
QIAasympphony DSP DNA Mini Kit (192)	For 192 preps of 200 µL each: Includes 2 reagent cartridges and enzyme racks and accessories	937236
QIAasympphony DNA Mini Kit (192)	For 192 preps of 200 µL each: Includes 2 reagent cartridges and enzyme racks and accessories	931236
QIAasympphony DSP Circulating DNA Kit (192)	Reagent cartridges, accessories and proteinase K vials for 192 preps of 2000 µL or 4000 µL each	937556
RNase A (17,500 U)	2.5 mL (100 mg/mL; 7000 units/mL, solution)	19101
Buffer ATL (4 x 50 mL)	4 x 50 mL lysis buffer	939016

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Revision	Description
R1, April 2022	Initial Release
R2, March 2023	Added "Sample Types & Layout in Protocol: EGFR mutation assessment by qPCR on Rotor-Gene Q instrument. Edited according to new brand template.

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